The Role of the Polyunsaturated Aldehydes in the Physiology and Ecology of Diatoms

Thesis

The Role of the Polyunsaturated Aldehydes in the Physiology and Ecology of Diatoms

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Abstract

In the last decade diatoms have been shown to release a wide range of secondary metabolites, such as polyunsaturated aldehydes (PUAs), produced by a wound-activated mechanism. PUAs are highly reactive molecules that in most cases induce a drastic reduction in the reproductive response of predators, such as copepods.

The variable toxicity of diatoms observed in nature suggests a modulation of PUA production by environmental factors. My results indicate a strong dependence of PUA production on culture age, nutrient limitation and other stress factors, such as UV exposure and low light conditions, in cultures of *Skeletonema marinoi*, suggesting a direct link between toxin production and cell physiological state.

Since it has been hypothesized that PUAs may be released in seawater also as a result of cell mortality, phytoplankton lysis rates were estimated during four oceanographic cruises conducted during periods of diatom blooms in the Northern Adriatic Sea. High lysis rates were observed and preliminary results indicated substantial amounts of PUAs released in seawater. The effects of dissolved PUAs were therefore investigated on organisms other than predators, such as co-occurring bacteria and phytoplankton in culture. PUAs are toxic for several phytoplankton species at concentrations well within the range potentially produced by diatoms. Diatoms can be immunized by sub-lethal concentrations of PUAs. In this case, PUAs may act as signal molecules for bloom termination. PUAs induce also different effects on bacterial growth; some strains are inhibited while others show remarkable resistance to these compounds, and a few of them were even stimulated.

Therefore, PUAs appear to have multiple functions in diatoms, namely as chemical defense against grazers, allelochemicals against phytoplankton and bacteria, and signal molecules within their populations.

CHAPTER 1

State of Art and Thesis Objectives

I. General introduction

To the casual observer, the ocean is a vast homogenous body of water. Beneath its surface are billions of tiny free-floating microscopic animals and plants collectively called plankton, a word derived from the Greek *planktos* meaning "wandering". The plankton may be defined as a carpet of drifting organisms that float more or less passively with the currents and are therefore at the mercy of water movements. The plankton has traditionally been divided into two main groups, the zooplankton and the phytoplankton. The former includes "myriads of animals" that live permanently in a floating state (i. e. holoplankton), and countless numbers of larvae and eggs of the animal benthos (sessile, creeping, and burrowing organisms) (i. e. meroplankton). Phytoplankton comprises all of the floating microscopic algae and bacteria, such as diatoms, dinoflagellates, coccolithophores, and cyanobacteria, capable of elaborating complex organic substances from the simple inorganic compounds dissolved in the water.

Phytoplankton species are at the base of the food web in the oceans and are by far the most important marine organisms able to produce life-sustaining O_2. Phytoplankton plays also a key role in the global carbon cycle and in the regulation of

the climate by controlling the amount of the greenhouse gas CO_2 in the atmosphere. Together with the "solubility pump" which is controlled by chemical and physical processes the "biological pump" helps to maintain a sharp gradient of CO_2 between the atmosphere and the deep ocean (see Chisholm 2000) (Figure I-1). Using sunlight for energy and dissolved inorganic nutrients, phytoplankton converts CO_2 to organic carbon, which forms the base of the marine food web. When phytoplankton are consumed by zooplankton (or when attacked by pathogens like viruses, or are dying for other causes), most of the organic carbon is converted back into CO_2 and released in the atmosphere. Another part of the organic carbon is trapped in bottom waters or in sediments for long periods of time (10s – 1000s of years). Global CO_2 fixation is approximately equal between terrestrial contributions and aquatic primary producers (Field et al. 1998). In some regions of the oceans, such as the Peruvian upwelling, annual primary production can reach 2 kg C m^{-2}, which is equivalent to the terrestrial production of cereal or corn crops (Field et al. 1998).

Of the marine organisms that are able to perform photosynthesis, diatoms are by far the most important. They are responsible for approximately 50% of marine primary production and it is estimated that 20% to 25% of all organic carbon fixation on the planet is carried out by diatoms (Nelson et al. 1995). Due to their unambiguous importance in the carbon cycle and for life on earth, many studies have been conducted to determine the factors responsible for their ecological success (Guillard and Kilham 1977).

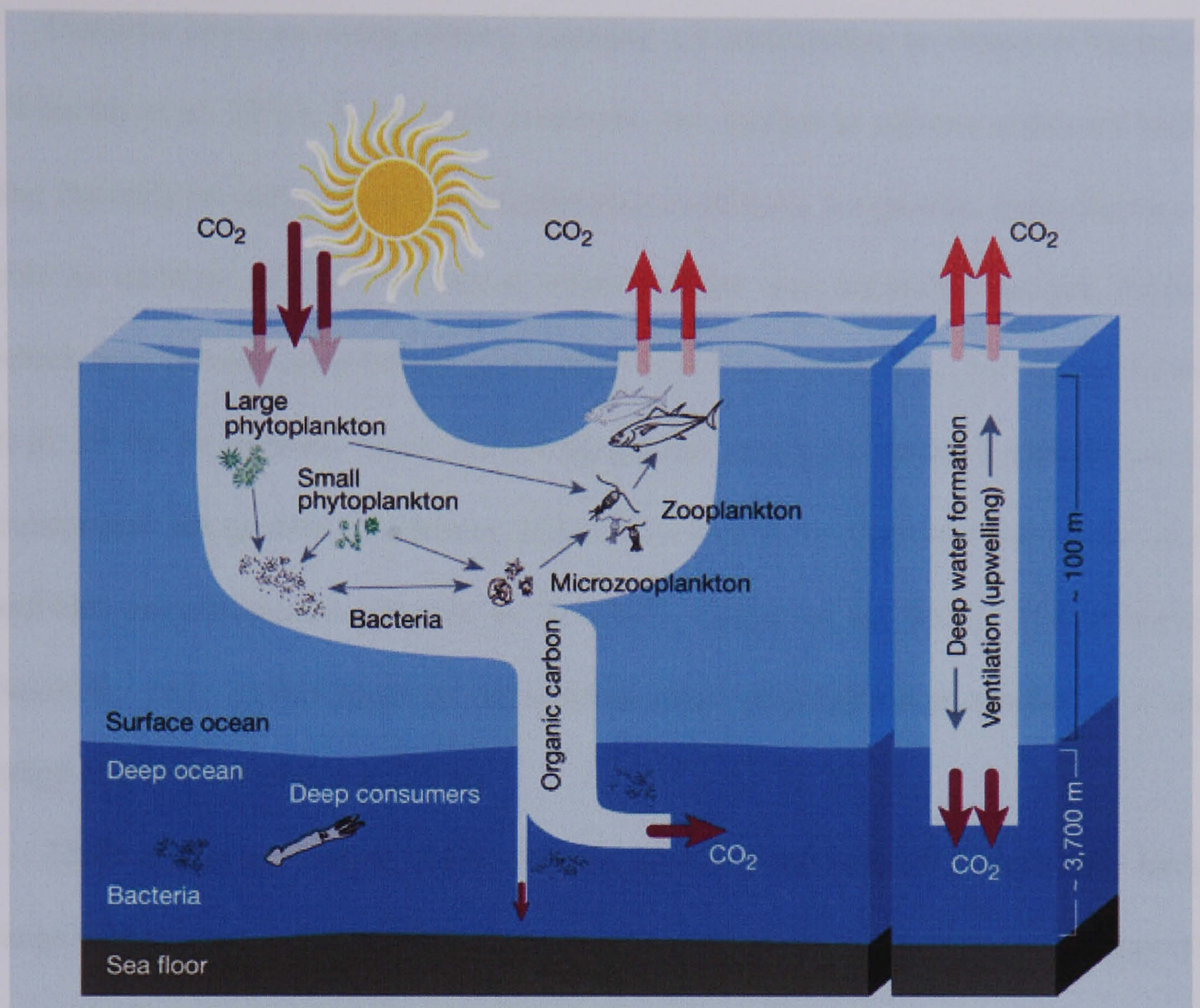


Figure I-1: The carbon cycle in the ocean. The flux of CO_2 from the atmosphere to the deep ocean is mediated by physical and chemical processes and by the "biological pump". This is defined as the export of organic carbon, produced by phytoplankton photosynthesis, from the surface to the deep ocean (from Chisholm 2000).

Diatoms have an extraordinary capacity of acclimation to resource limitations (Wilhelm et al. 2006). While their relatively low surface to volume quotients implies that the cells are well adapted to nutrient-rich conditions for growth, most diatoms are able to undergo cell size reduction when nutrient concentrations become limiting, which may provide an efficient acclimation to critical conditions (for review Sarthou et al. 2005). In addition, many studies report that both high- and low-affinity nutrient transporters are present in diatoms, which allows them to adapt nutrient uptake to the ambient concentrations (Donald et al. 1997; Collos et al. 2005). This metabolic flexibility may enable them to outcompete other phytoplankton species in a wide range of environmental conditions.

There is also growing evidence that the silicified cell wall of diatoms may have a range of functions and thereby would provide an ecological advantage with respect to non-silicified phytoplankton cells. For instance, the silica wall may serve as regulator of sinking rate, as an effective pH buffer which facilitates the enzymatic conversion of bicarbonate to CO_2, and as an ultraviolet filter (reviewed in Raven and Waite 2004). It has been also proposed that the strong cell wall may act as a protection against predators and pathogen attack (Smetacek 2001). Hamm et al. (2003) demonstrated that the shape, size and strong silicified cell wall provide an effective first line of defense against copepods. The diatom cell can resist to pressure up to 100-700 tonnes m^{-2} before collapsing, depending on their size and where the pressure is applied (Hamm et al. 2003). Since large copepods are able to apply such pressure on the cell wall, silification will offer therefore a good protection against small copepods. The thickness of the cell wall can also increase in response to attack by large copepods,

such as *Calanus helgolandicus* (Pondaven et al. 2007), indicating that silicification in diatoms is not only a constitutive mechanical protection for the cell, but also a phenotypically plastic trait modulated by grazing.

Since the silicified cell wall may restrict the access of protoplast, it has been proposed that silicification provides a mechanical protection against virus attack (Smetacek 1999). However, only few viruses are known to be able to infect phytoplankton (Suttle 2005), and there is recent evidence that at least a few diatoms can be infected by viruses (Nagasaki et al. 2004, 2005; Bettarel et al. 2005). Therefore, the silicified cell wall does not offer a complete protection against viruses. In addition, the infection of eukaryotic parasites in diatoms, such as heterotrophic dinoflagellates, has been known for a long time and does not support the hypothesis of a role of the silicified cell wall in restricting infection by diatoms (see Raven and Waite 2004).

Besides their physiological and morphological responses to changing environmental factors, there is growing evidence that diatoms may use a chemical defense system, which can potentially affect both predators and competitors (e.g. Pohnert 2004). The demonstration of an ecologically relevant chemical defense of phytoplankton is currently a major challenge, partly due to the fact that interactions are very complex and occur among numerous microscopically small species, and to technical difficulties in the identification and quantification of the noxious molecules (Wolfe 2000). The following discussion summarizes our sparse knowledge about chemical defense in marine plankton and outlines the role that defensive metabolites can play in this ecosystem.

II. Secondary metabolites in marine phytoplankton

To date, about two hundred phytoplankton species, belonging to various groups, such as dinoflagellates, diatoms, cyanobacteria, and prymnesiophytes, are known or suspected to induce detrimental or stimulatory effects on a wide spectrum of organisms (Landsberg 2002). Many microalgae produce a variety of different, often unique, molecules which are released in the environment (Hay 1996). These chemicals are referred as secondary metabolites and are not directly involved in building up the machinery of life. Often secondary metabolites constitute a very small fraction of the total biomass of an organism, and it is not always clear what biological role these compounds play. Cembella (2003) defined chemical ecology as "the relationship between the structure and function of metabolites and how these affect organisms in the environment, controlling the coexistence and coevolution of species". These chemical ecological interactions have been extensively studied in terrestrial plants due to the economic importance of agricultural systems, and are known to contribute to defense mechanisms against grazers and disease organisms such as parasites (Inderjit and Duke 2003). In marine ecosystems, much attention has focused on chemical ecological interactions within and among invertebrate species, such as sponges, echinoderms and polychaetes, particularly in benthic tropical ecosystems, where species diversity and resource competition are expected to be high (Hay and Fenical 1996). The chemical ecology of marine microalgae has received less attention than that of terrestrial plants and invertebrate fauna, mainly due to technical difficulties in measuring interactions between physical, chemical and biological factors at different

time and space scales (Wolfe 2000). Although the function of most microalgal secondary metabolites remains mostly uninvestigated, there is growing evidence that they may play an important role for algal bloom development, dynamics, and fate (Landsberg 2002). A number of studies suggest that some phytoplankton species may produce secondary metabolites as a chemical defense by affecting growth and physiological performance of bacteria and competitor species (Legrand et al. 2003), and/or by acting as feeding deterrents which restrict predation (Tillmann 2004).

II.1. Secondary metabolites as allelochemicals

Secondary metabolites produce by one species that induce negative effects on the physiology of another species are called allelochemicals, or allelopathic chemicals, derived from the Greek *allelon* and *pathos* meaning "to suffer reciprocally". Although the word allelopathy refers to a negative effect, Molish (1937) enlarged the definition to both inhibitory and stimulatory chemical interactions between and among all types of plants and to also include effects on microorganisms. This definition includes any non-nutritional compounds produced by one organism that affect the growth, behaviour, health or population biology of other species. Willis (1985) presents six criteria for the demonstration of allelopathy: (1) a consistent pattern of inhibition of the target species must be seen; (2) the inhibitor species must produce a toxic chemical; (3) a mechanism for toxic chemical release into the environment must be present; (4) there must be a system for transporting or accumulating the toxic chemical

within the environment; (5) the target species must have some means of taking up the toxic chemical; and (6) the pattern of inhibition must not be explainable by physical factors or other biotic factors, especially competition and grazing. The ideal concept of allelopathy is probably very difficult to demonstrate since it involves showing that the allelopathic effects observed persist when other factors, such as nutrient limitation or resource competition, are eliminated (Inderjit and Del Moral 1997). However, since in natural systems both resource competition and allelopathy can occur simultaneously, it is very difficult to distinguish the effect of each mechanism. The relative contribution of each mechanism is affected by factors that enhance one or the other mechanism, like in terrestrial plants, where nutrient limitation has been shown to influence allelopathic interactions by modulating the production of allelochemicals and by making the target organisms more sensitive to them (Reigosa et al. 1999).

In aquatic systems, the biochemical and physiological mechanisms of how stress modulates the allelopathic effect of phytoplankton are not well understood, due to the lack of information on the chemical nature and synthetic pathways of many allelochemicals. Nevertheless, the production of toxins, which could potentially act as allelochemicals, is strongly influenced by their growth conditions. For example, the production of saxitoxin by the dinoflagellate *Alexandrium* increases in the exponential phase of growth and under phosphorus limitation, while it decreases under nitrogen limitation (Anderson et al. 1990). Domoic acid is produced in higher quantities by the diatom *Pseudo-nitzschia* spp. during stationary growth phase and under silicon and phosphorus limitation but not under nitrogen limitation (Pan et al. 1998).

Dimethylsulfoniopropionate produced by the diatom *Thalassiosira pseudonana* increases to high values in the exponential phase and under nutrient-limiting conditions (Bucciarelli and Sunda 2003). In addition, it has been shown that N-and P-limitation enhance the sensitivity of target species to extracts of the toxic prymnesiophyte *Prymnesium parvum* (Fistarol et al. 2005). Therefore, under nutrient limiting conditions, it is likely that the allelopathic effect will be higher due to both increased toxin production and increased sensitivity of the target species. In such situations, as at the end of a bloom, the competitive balance will turn toward the allelopathic species, which will have an advantage. Despite the fact that these observations highlight the putative role of phytoplankton-derived toxins as allelochemicals, there are only few studies that provide evidence of such a role in aquatic ecosystems, mainly due to the technical difficulties mentioned above.

In freshwater environments, most of the studies on allelopathy concern toxic cyanobacteria (Lansberg 2002). Keating (1977) presents evidence of a direct role of allelopathy in algal succession by combining three years of field observations and laboratory experiments using axenic cultures. He reported that cell-free filtrates of dominant cyanobacteria from a freshwater lake inhibit the growth of predecessor species and promote the growth of the successor. These observations suggest that algal allelopathy is one of the controlling factors in bloom dynamics. Another study showed in vivo that the alternating dominance of the dinoflagellate *Peridinium gatunense* and the cyanobacterium *Microcystis* sp. may be caused by reciprocal, density-dependent allelopathic interactions (Vardi et al. 2002).

In the marine environment, although the possibility of allelochemical production has been less explicitly studied, there is some evidence that allelopathy may play an important role in algal succession and competition (Legrand et al. 2003). Table I-1 summarizes the most relevant published reports of marine microalgae, including cyanobacteria, known to produce allelochemicals against other algae, mostly based on crossed-culture techniques and cell filtrate exposure. Pratt (1966) observed that the phytoplankton community in Narragansett Bay was alternatively dominated by blooms of the diatom *Skeletonema costatum* and the dinoflagellate *Olisthodiscus luteus*. With *in vitro* and *in situ* experiments, they showed that *O. luteus* achieved dominance by producing a tannin-like substance that had an inhibitory effect of *Skeletonema costatum* at high concentrations but stimulated the growth of this diatom at lower concentrations. The authors suggested that this may explain the alternating dominance of these two species. Another example is the occurrence of the monospecific bloom of the domoic acid-producing diatom *Pseudo-nitzschia pungens* in Cardigan Bay simultaneously with another diatom *Rhizosolenia alata* in Hillsborough River estuary (Subba Rao et al. 1995). These authors reported that the growth *R. alata* and *P. pungens* were reciprocally suppressed (*R. alata* by domoic acid, and *P. pungens* by *R. alata* cell-free extracts) and hypothesized that allelopathy may therefore play a role in algal succession. Yamasaki et al. (2007) observed the same phenomena between *S. costatum* and the raphidophyte *Heterosigma akashiwo*.

Table I-1: Examples of marine phytoplankters that may have an allelopathic effect on other microalgal species.

Clade	Producers	Chemicals	Competitors	Relevant references
Cyanobacteria	*Anabaena* *Fischerella* *Microcystis* *Nostoc* *Nodularia*	Anatoxin Cyanobacterin Microcystin Uncharacterized molecules	Chlorophytes Chrysophytes Cryptophytes Cyanobacteria	Pushparaj et al. 1998 Nagle and Paul 1999 Landsberg 2002 Suikkanen et al. 2004,2005 Valdor and Aboal 2007
Diatoms	*Pseudonitzschia* *Skeletonema* *Thalassosira*	Domoic acid Uncharacterized molecules	Diatoms Raphidophytes	Sharp et al. 1979 Subba Rao et al. 1995 Yamasaki et al. 2007
Dinoflagellates	*Alexandrium* *Coolia* *Dinophysis* *Gymnodinium* *Heterocapsa* *Karenia* *Olisthodiscus* *Prorocentrum* *Peridinium*	Brevetoxin Ichthyotoxin Maitotoxin Okadaic acid Saxitoxin Tannin-like Uncharacterized molecules	Chlorophytes Diatoms Dinoflagellates	Pratt 1966 Windust et al. 1996 Arzul et al. 1999 Sugg and Van Dolah 1999 Donner et al. 2000 Rengefors and Legrand 2001 Tillmann and John 2002 Fistarol et al. 2004 Kubanek et al. 2005
Prymnesiophytes	*Chrysochromulina* *Prymnesium*	Ichthyotoxin Prymnesin Uncharacterized molecules	Diatoms Dinoflagellates Diatoms Silicoflagellates Raphidophytes Euglenophytes Cryptophytes Prasinophytes	Schmidt and Hansen 2001 Fistarol et al. 2003; Granéli and Johansson 2003a Barreiro et al. 2005

Allelopathic interactions are also found in cyanobacteria, such as *Nodularia* sp. that induces strong allelopathic activity against other cyanobacteria and diatoms (Pushparaj et al. 1998; Suikkanen et al. 2004, 2005); in dinoflagellates, with *Karenia brevis* that has a growth-inhibitory effect on other dinoflagellates and diatoms (Schmidt and Hansen 2001) and stimulatory effect on cryptophytes (Kubanek et al. 2005); and in prymnesiophytes, such as *Chrysochromulina polylepis* and *Prymnesium parvum* that have algicidal properties on a wide range of phytoplankton species (Granéli and Johansson 2003b; Fistarol et al. 2003). However, in most of these cases, allelochemicals have not been identified and may be different from already known toxins. For instance, okadaic acid, produced by the dinoflagellate *Prorocentrum lima* has a potent growth inhibitor effect on other microalgae (Windust et al. 1996). However, in a detailed study, Sugg and Van Dolah (1999) demonstrated that, although okadaic acid has a growth-inhibitory potential against other phytoplankton and epiphytic algae, it is not the major contributor to the allelochemical activity of exudates from *P. lima*. Cell-free filtrates of *K. brevis* have potent growth inhibition effects on several algal species while brevetoxin accounts for modest inhibition by *K. brevis* of only one algal species (Kubanek et al. 2005).

There are also indications that phytoplankton may produce allelochemicals against heterotrophic bacteria. There are diverse relationships between phytoplankton and bacteria in marine ecosystems. One of them is a nutritional relationship. Bacteria utilize various organic compounds produced by phytoplankton and play a major ro1e in the regeneration of inorganic nutrients. Another important relationship is the mutual

control of biological activities by external metabolites. There are many reports of aquatic bacteria that cause algal cells to lyse, such as a vibrio-shaped bacterium on the chlorophyte *Chlorella* sp. (Cole 1982) and the *Cytophaga* sp. on the diatom *Skeletonema costatum* (Mitsutani et al. 1992).

Turning to effects of algae on bacteria, Pratt (1948) isolated from *Chlorella vulgaris* a compound named chlorellin, which was effective against pathogenic bacteria when it was excreted in the culture medium. Since this study, antibacterial effects have been noticed in other algal species (Table I-2). For example, extracts of the dinoflagellate *Alexandrium tamarense* induced a dramatic antibiotic activity against the pathogenic bacterium *Staphylococcus* and exhibited simultaneously both inhibitory and stimulatory effects on mixed populations of marine bacteria (Burkholder et al. 1960). The prymnesiophyte *Prymnesium parvum* produces acrylic acid, via the breakdown of dimethylsulfoniopropionate, which can suppress bacterial growth during the algal exponential growth phase, but loses the ability to produce the toxin at inhibitory concentrations as the bloom ages, allowing growth of bacteria (Khailov 1968). Eberlein et al. (1985) observed a similar pattern in cultures of *Phaeocystis pouchetti*. The diatoms *Skeletonema costatum*, *Chaetoceros socialis* and *Phaeodactylum tricornutum* are well-known species for their antibacterial properties against a broad spectrum of aquacultural pathogens (Naviner et al. 1999 and references therein). It has been observed that *S. costatum* can have both an inhibitory and stimulatory effect on marine bacteria (Bell and Mitchell 1972; Bell et al. 1974; Kogure et al. 1979).

Table I-2: Examples of marine phytoplankton species that may have an allelopathic effect on bacteria.

Clade	Producers	Chemicals	Bacteria	Relevant references
Chlorophytes	*Chlorella*	Chlorellin	Human pathogens	Pratt 1948
Cyanobacteria	*Nodularia* *Phormidium*	Uncharacterized molecules	Bacteria isolated from waste treatment	Pushparaj et al. 1998 Kirkwood et al. 2006
Diatoms	*Chaetoceros* *Phaeodactylum* *Skeletonema*	Uncharacterized molecules	Aquacultural pathogens	Bell and Mitchell 1972 Bell et al. 1974 Kogure et al. 1979 Naviner et al. 1999
Dinoflagellates	*Alexandrium* *Prorocentrum*	Norcaroteno Uncharacterized molecules id	Human pathogen Marine bacteria population	Burkholder et al. 1960 Trick et al. 1984
Prymnesiophytes	*Phaeocystis* *Prymnesium*	Acrylic acid	Pathogenic bacteria Intestinal microflora	Sieburth 1960 Khailov 1968 Eberlein et al. 1985

Beside the fact that no evidence has been found to demonstrate that these effects may be related to the production of allelochemicals by the algae, most of the studies have focused on pathogens and not on co-occurring marine bacteria, therefore the question about their ecological relevance still remains.

II.2. Secondary metabolites as chemical defense against grazers

In addition to the above-mentioned effects on microalgae and bacterial physiology, it has been shown that secondary metabolites produced by phytoplankton can have also adverse effects on grazers (Table I-3). When the dinoflagellate *Gymnodinium breve* is fed to copepods, a paralyzing effect and elevated heart rate is observed in most adults and a loss of the neuromuscular control in nauplii. Ianora et al. (1999) report reduced sperm quality in copepods fed certain dinoflagellate diets, but it remains unclear whether this is due to nutrient deficiency or related to toxin production. It has been suggested that the dinoflagellate *Gyrodinium aureolum* greatly reduces egg production in copepods due to nutritional deficiency rather than toxin production (Irigoien et al. 2000). Mixed diets with different proportions of brevetoxin-producing *Karenia brevis* and a non-toxic dinoflagellate decreased the survivorship of the co-occurring copepod *Acartia tonsa* with increasing proportion of *K. brevis* (Prince et al. 2006). However, *K. brevis* extracts did not affect the copepods which exclude a direct function of the brevetoxins in the observed toxicity.

Table I-3: Examples of marine phytoplankton species using potentially chemical defense against predators

Clade	Producers	Chemicals	Predators	Relevant references
Cyanobacteria	*Anabaena* *Nodularia* *Trichodesmium*	Saxitoxin Malyngamide Ypaoamide Microcystin Uncharacterized molecules	Copepods Cladocerans Rotifers Brine shrimps	Turner et al. 1997 Pushparaj et al. 1998 Nagle and Paul 1999
Diatoms	*Chaetoceros* *Phaeodactylum* *Pseudo-nitzschia* *Skeletonema* *Thalassosira*	Aldehydes Apofuco-xanthinoids Domoic acid	Copepods Krill Sea urchins	Ianora and Poulet 1993 Uye 1996 Shaw et al. 1997 Ban et al. 1997 Miralto et al. 1999 Caldwell et al. 2003; Ianora et al. 2004 Bargu et al. 2006
Dinoflagellates	*Alexandrium* *Dinophysis* *Gymnodinium* *Heterocapsa* *Karenia* *Prorocentrum*	Brevotoxin Okadaic acid Saxitoxin Uncharacterized molecules	Heterotrophic dinoflagellates Ciliates Copepods Brine shrimps Rotifers	Turner and Tester 1997 Ianora et al. 1999 Guisande et al. 2002 Tillmann and John 2002 Turner and Borkman 2005 Prince et al. 2006
Prymnesiophytes	*Chrysochromulina* *Emiliania* *Phaeocystis* *Prymensium*	Dimethysulfide Primnesin Uncharacterized molecules	Ciliates Copepods Heterotrophic dinoflagellates Brine shrimps Rotifers	Wolfe et al. 1997 Tillmann 2003 Granéli and Johansson 2003a Strom et al. 2003a, 2003b Houdan et al. 2004

The situation becomes more complex if the reproductive success of the copepods is also considered. Predator reproductive success depends on egg production rate, egg hatching and naupliar survival. Thus, toxins that affect these processes provide a strategy for an indirect chemical defense. In the experiments with *A. tonsa*, egg production rates decreased with increasing proportion of *K. brevis* in the food, but egg-hatching success remained constantly high. Decreased egg production could not be correlated to toxicity and thus adverse effects were probably due to inadequate food quality of this alga (Prince et al. 2006). Similar ambiguous effects have been observed in the filamentous toxic cyanobacteria of the genus *Trichodesmium* (Turner and Tester 1997) and these effects may rarely occur in nature due to the presence of many other "good quality" food sources that copepods could preferentially select (Turner and Borkman 2005). These examples demonstrate that, even with a simplified scenario of two species, it is difficult to separate unambiguously the impact of toxicity and food quality.

Some diatoms, which are traditionally considered as optimum nutritional food for zooplankton (Parsons et al. 1984b), produce domoic acid that has been shown to induce reduction in fecundity and cell death in the copepod *Tigriopus californicus* at low concentrations (Shaw et al. 1997), while Lincoln et al. (2001) did not observe any adverse effect with *Temora longicornis*. Bargu et al. (2006) observed that grazing rate of the krill *Euphausia pacifica* is greatly decreased by domoic acid, but at concentrations ten times higher that those found in nature (Doucette et al. 2002). Thus, it is apparent that effects depend on the types of assays performed, the taxa involved and the concentrations used. Even when nutrient deficiency can very likely be

excluded from the detrimental effect on grazers, the understanding of the role of toxins as chemical defense is not straightforward.

Another defensive strategy employed by plants as protection against grazer is an activated enzyme-cleavage mechanism of defense. The general pattern involves a precursor compound(s) of the active molecule stored in a non-toxic way. Upon damage or stress to the algae by grazing, the precursor molecules are enzymatically converted to the active compound. Many marine macroalgae have been shown to use extensively activated defense systems (Van Alstyne et al. 2001), and there is evidence of such mechanisms in phytoplankton (Pohnert 2004).

Ianora and Poulet (1993) found that a diatom diet of *Thalassosira rotula* induced low hatching success in copepods though high egg production was observed. Uye (1996) observed that copepod nauplii that did hatch from diatom-fed females were generally deformed, expressing marked morphological asymmetry, and died a few days after hatching. Poulet et al. (1994) proposed that the detrimental effect of diatoms on copepod reproduction was caused by the presence of antimitotic compounds that could accumulate in the reproductive organs of the copepods and be incorporated into the eggs during vitellogenesis. Miralto et al. (1999) observed that the hatching success of wild copepods feeding on a diatom-dominated bloom was also strongly reduced. These authors showed that the effect was caused by short chain polyunsaturated aldehydes, which are produced seconds after cell membrane disruption by grazers (Pohnert 2000). Deleterious effects of these compounds on reproductive processes

have since then been demonstrated in other organisms, such as echinoderms, polychaetes, ascidians and crustaceans (Caldwell et al. 2003 and reference therein).

Recently, it has been shown that other compounds are also produced together with polyunsaturated aldehydes (d'Ippolito et al. 2005; Wichard and Pohnert 2006; Fontana et al. 2007a), such as fatty acid hydroperoxides which have been recently shown to have negative effect on copepod reproduction (Fontana et al. 2007b). Therefore, it is likely that the effect of aldehydes and hydroperoxides may act additively, as predicted by Faust et al. (2001), or perhaps even synergistically. Such chemical defense based on the production of different classes of molecules might provide a more efficient defense against grazer than a mechanism based on a single class of molecules.

A similar example of an activated enzyme-cleavage mechanism of defense in phytoplankton has been reported for the bloom-forming coccolithophorid *Emiliania huxleyi*. This alga produces the gas dimethylsulfide and the feeding deterrent acrylate via the cleavage of dimethylsulfoniopropionate that occurs immediately after cell injury by grazers (Wolfe et al. 1997). In feeding assays with a protist grazer, these authors showed selective feeding on a strain with low dimethylsulfoniopropionate activity compared to one with higher activity. The prymnesiophyte *Phaeocystis* is an important bloom forming genus producing dimethylsulfide and acrylate and is thought to be a poor food source for a variety of zooplankton grazers. Turner et al. (2002) concluded that although copepods feed well upon *Phaeocystis*, the resulting poor fecundity on this diet may inhibit copepod population increases during blooms, and therefore may contribute to the perpetuation of blooms. *Phaeocystis pouchetii* has recently been reported to also produce the polyunsaturated aldehyde decadienal

(Hansen et al. 2004a) indicating that chemical defense against grazer may likely be insured by more than one chemical.

II.3. Secondary metabolites as signalling molecules

A recent line of research is highlighting the role of secondary metabolites as information-conveying molecules in cell-to-cell signalling, so called semiochemicals (Vos et al. 2006). There is clear evidence in terrestrial ecosystems that when attacked by grazers, plants can produce secondary metabolites that attract the predators of these grazers (Agrawal 2000). Several authors have suggested that similar tritrophic defensive interactions may also occur in aquatic ecosystems (see Wolfe 2000): Steinke et al. (2002) suggest that the volatile dimethylsulfide that is released during grazing by microzooplankton on phytoplankton can attract mesozooplankton copepods, thus enabling them to more efficiently graze on microzooplankton. In addition, some pelagic seabirds can sense dimethylsulfide (Nevitt et al. 1995) and may enable them to locate zooplankton-rich areas and eat on fishes which could decrease the predation pressure on mesozooplankton. These complex interactions would indirectly decrease the grazing pressure on phytoplankton. However, this picture of a putative marine indirect defense is simplified and still seems a bit controversial. Attraction of copepods to dimethylsulfide is suggested, but not shown in Steinke et al. (2002) and it is not straightforward whether the resulting complex, multitrophic food web interactions actually protect the algae from grazing.

III. Diatom-derived polyunsaturated aldehydes

The traditional view that diatoms are a high quality food boosting secondary production during blooms has recently been challenged in the paper "paradox of diatom-copepod interactions" (Ban et al. 1997). These authors examined the effect of seventeen diatom species on sixteen species of copepods. Of these seventeen, only one did not result in reduced egg production rates in comparison with dinoflagellate diets. Testing different diatom-copepod combination, diatoms reduced on average fecundity by 90% and hatching success by 80%. Whereas the bulk of evidence for diatom toxicity on copepods is based on laboratory studies, Miralto et al. (1999) present evidence from the field showing that the hatching success of wild copepods feeding on a diatom-dominated bloom in the Northern Adriatic Sea is also heavily reduced, with only 12% of the eggs hatching compared with 90% in post-bloom conditions.

The causative agents of diatom-derived embryonic inhibition were first isolated and identified by Miralto et al. (1999) from *Thalassosira rotula*, *Skeletonema marinoi* (previously misidentified as *S. costatum*, see Sarno et al. 2005) and *Pseudo-nitzschia delicatissima* as two C10 short chain polyunsaturated aldehydes (abbreviated as PUAs); 2E,4E/Z-decadienal and 2E,4E/Z,7Z-decatrienal (Figure I-2). These findings have been further confirmed by Pohnert (2000, 2002), d'Ippolito et al. (2002a, 2002b, 2003) and Wichard et al. (2005b) using different methodologies, and identifying a more complete range of PUAs from *S. marinoi* and *T. rotula*. The additional aldehydes are: 2E,4E/Z-heptadienal, 2E,4E/Z-octadienal, and 2E,4E/Z,7Z-octatrienal (Figure I-

2). PUA production has been examined in 51 diatoms, including 71 isolates, and it has been shown that their production is species and strain dependent. Twenty seven of the cultivated isolates examined, representing 38% of the investigated isolates, released PUAs in concentrations from 0.01 to 9.1 fmol per cell.

Among PUAs, 2E,4E-decadienal has been widely used as a model aldehyde to show deleterious effects on the reproduction of several marine invertebrates, such as echinoderms, polychaetes, ascidians, crustaceans and molluscs (Caldwell et al. 2003). Adolph et al. (2003) determined that the inhibitory activity of PUAs is not restricted to 2E,4E/Z-decadienal and 2E,4E/Z,7Z-decatrienal, but rather dependent on the reactive Michael acceptor element (Figure I-3). In addition, Adolph et al. (2003) showed that both 2E,4Z- and 2E,4E-dienals have similar activity, Therefore, although the biological activity of other PUAs, such as 2E,4E/Z-heptadienal, 2E,4E/Z-octadienal, and 2E,4E/Z,7Z-octatrienal, have not been yet tested, it is very likely that they contribute to the negative effect observed on grazers. Molecules belonging to this class are unstable and react with nucleophiles (attracted to positive nuclear charge) and they are thus often associated with toxicity (Van Iersel et al. 1997; Refsgaard et al. 2000). It can therefore be concluded that no specific receptor or enzymatic activity is affected by the diatom-derived toxicity of PUAs, but rather that unspecific interactions of unsaturated aldehydes are responsible for their activity (Adolph et al. 2003, 2004).

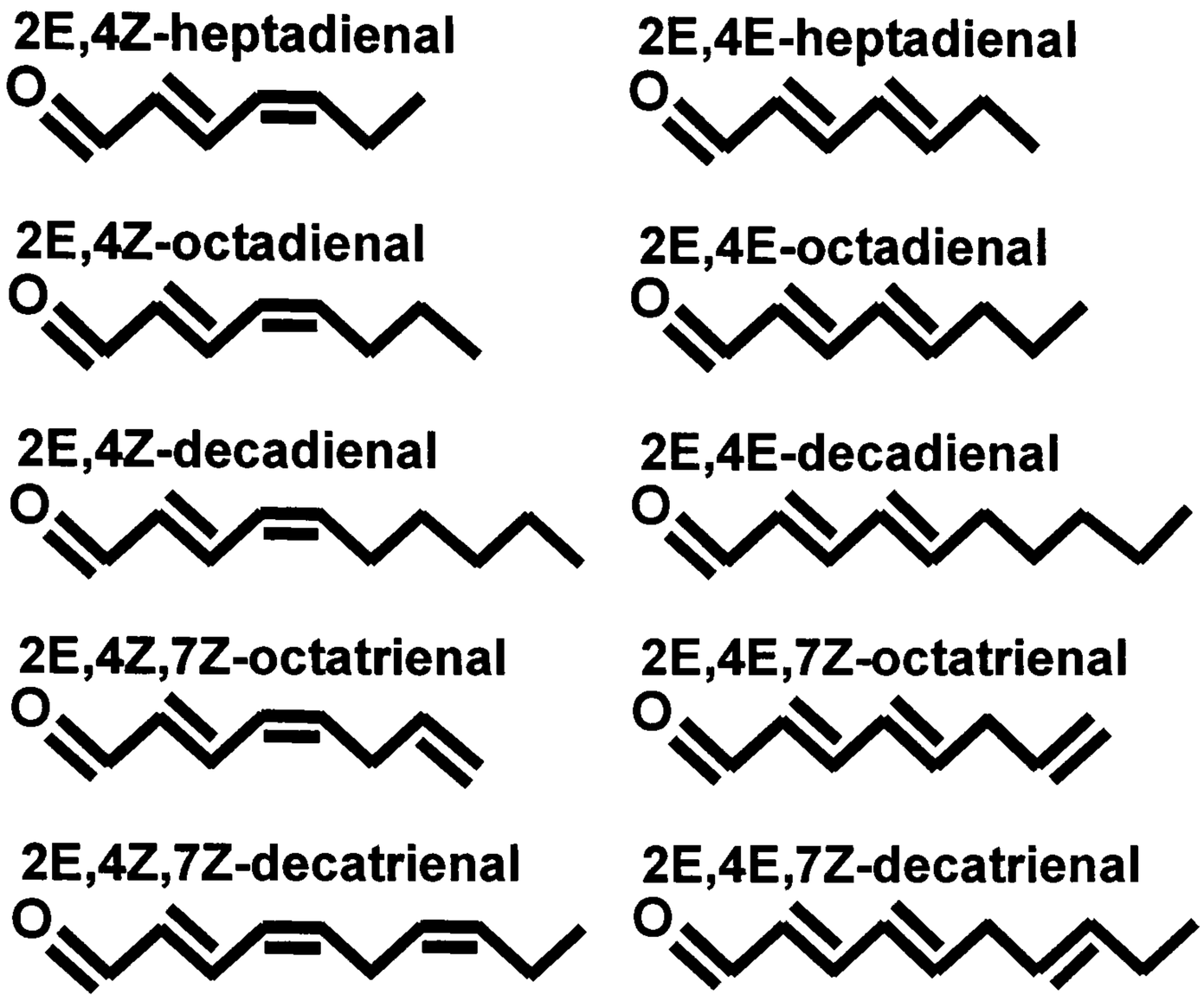
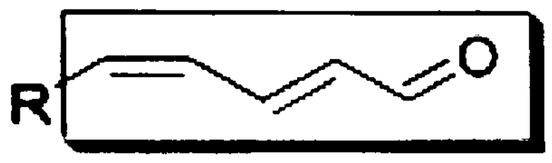


Figure I-2. Structure of diatom-derived polyunsaturated aldehydes.



R = alkyl, alkenyl, acid

Figure I-3: Structure of the reactive Michael acceptor element (framed) found in anti-proliferative metabolites from diatoms. This element can react with a wide range of nucleophiles.

Pohnert (2000) proposed that the formation of PUAs in *Thalassosira rotula* is a wound activated mechanism. Quantification experiments with intact diatom cells did not result in any detectable PUA production. This situation changes dramatically after seconds after mechanical damage to the algae. For example, *T. rotula* releases PUAs seconds after cell disruption and saturation levels in culture in the range of 5 fmol of PUA per cell are reached within 2 minutes (Pohnert 2002, Fontana et al. 2007a). A comparable dependence of PUA production on cellular disintegration or osmotic stress has also been shown with preparations of freshwater diatoms (Wendel and Juttner 1996). This wound-activated chemical defense might be a way for diatoms to overcome several limitations. On the one hand, it avoids the cost of continuous toxin production and release (Juttner 1999). On the other hand, it minimizes the risk of self poisoning that might occur if defensive metabolites are stored in the cells. This risk is especially high in the case of the reactive 2E,4E-decadienal, which can unspecifically bind with DNA disturbing replication and mitotic events (Caravalho et al. 2000, Hansen et al. 2004b). A wound-activated defense mechanism thus permits investment in cellular resources during normal growth, and the production of defensive metabolites from these resources is only initiated upon demand.

The PUAs are oxylipins produced via a lipoxygenase-mediated pathway involving the degradation of free polyunsaturated fatty acids (here abbreviated as PUFAs) liberated by galacto- and phospholipase, which feed the downstream lipoxygenase leading to the production of hydroperoxides that in turn are transformed into PUAs by lipolytic acyl hydrolases (d'Ippolito et al. 2004) (Figure I-4). The bloom-forming

diatom *Skeletonema marinoi* produces the C_7 aldehyde 2*E*-4E/*Z*- heptadienal, as well as the C_8 aldehydes 2*E*-4E/*Z*-octadienal and 2*E*-4E/*Z*,7Z-octatrienal, which are derived from eicosapentaenoic, hexadecatrienoic, and hexadecatetraenoic acid, respectively (d'Ippolito et al. 2004), while in *Thalassosira rotula* 2E,4E/Z-decadienal and 2E,4E/Z,7Z-decatrienal derived from arachidonic and eicosapentaenoic acid, respectively (Pohnert 2005). In terrestrial plants, when the amount of oxylipins exceeds a certain threshold, lipoxygenase activity is inhibited (Spiteller 2003), suggesting that the process reaches a steady state between consumption of PUFAs and synthesis of oxylipins. In diatoms, oxylipin production, such as PUAs, is a continuous process, which is reinitiated when the product is removed from the enzyme (Fontana et al. 2007a). At sea, it is very likely that equilibrium is constantly disturbed since PUAs are washed away by diffusion, and therefore PUA production would go on until the total consumption of free PUA-precursor PUFAs. This may therefore contribute to maintaining high local PUA concentrations at sea. This strategy - building up and maintaining a high local concentration of defensive metabolites only in response to damage - allows targeting secondary metabolites very efficiently.

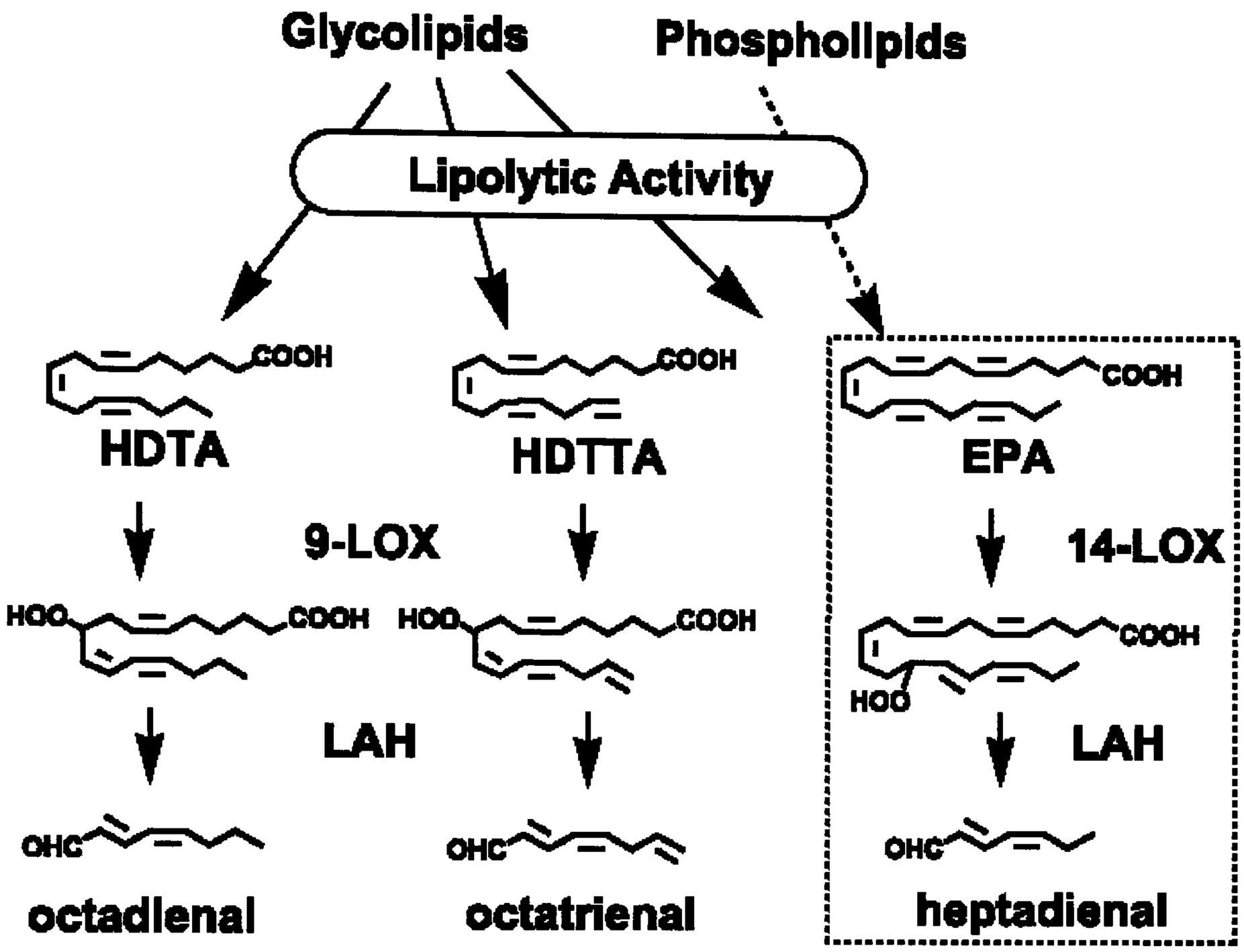


Figure I-4. Proposal for the biosynthesis of polyunsaturated aldehydes (octadienal, octatrienal and heptadienal) in the marine diatom *Skeletonema marinoi*. Hydrolysis of chloroplast-derived glycolipids leads to free forms of hexadecatrienoic acid (HDTA), hexadecatetraenoic acid (HDTTA) and eicosapentaenoic acid (EPA), which feed the downstream enzymes of the lipoxygenase (LOX) pathway. The lipolytic activity is also able to promote release of EPA from phospholipids (from d'Ippolito et al. 2004)

The question has arisen as to how diatoms regulate the rapid production of reactive PUAs. PUA production starts within seconds after wounding in seawater, a fact that makes regulation through transcription and *de novo* biosynthesis of the enzymes of the lipoxygenase pathway highly unlikely (Pohnert 2000). Since addition of precursor free PUFAs to wounded diatom leads to the increased formation of PUAs, Pohnert (2002) hypothesized that the production is apparently substrate limited. However, this increase of PUA formation can also be due to the fact that the equilibrium between free PUFAs and PUAs is disturbed after addition of extra PUFAs leading to reinitiate aldehyde production (see Fontana et al. 2007). Nevertheless, these observations highlight the importance of the availability of free PUFAs as a key factor for the formation of PUAs. Therefore, any mechanism that modulates the amount of free PUFAs could potentially influence the production of PUAs.

Since PUFAs are not stored in the free form but conjugated to phospho- and galactolipids (Pohnert 2002; d'Ippolito et al. 2004), mechanisms that modulate the activity of phospholipase A2 and galactolipases will affect the production of free fatty acids and subsequent oxylipins. In terrestrial plants, phospholipase A_2 activity is shown to be regulated by Ca^{2+} signalling and the degree of phosphorylation/dephophorylation of the enzyme, as well as the presence of reactive oxygen species and elicitors such as free fatty acids (Chandra et al. 1996; Chapman 1998; Narvaez-Vasquez et al. 1999; Wang 2001). Therefore, it is likely that these factors may also influence the production of PUAs in phytoplankton. In freshwater chrysophytes, light stress, iron, nitrogen and phosphorus limitation affect the production of PUAs, similar to those isolated from diatoms (Watson and Satchwill

2003). However, it remains unclear whether these environmental stresses influence the amount of PUA-precursor PUFAs, and/or enzyme activities. Such studies need to be undertaken.

The lipoxygenase pathway is apparently activated only in response to herbivore wounding and consequently PUAs have been considered only as a mechanism of chemical defense against grazers. However, in terrestrial plants, it has been shown that phospho- and galactolipase activities are also required for cell growth and proliferation (by building membrane lipids), and for senescence and apoptosis (by regulating loss of membrane integrity) (Chapman 1998). Spiteller (2003) suggests that oxylipins, such as lipid hydroperoxides and PUAs, may induce three types of physiological responses: moderate amounts of oxylipins would allow cell division and cell proliferation, while large and very large amounts of oxylipins would induce necrosis and apoptosis, respectively. It has been recently shown in the marine diatom *Thalassosira weissflogii* that large amounts of decadienal induce growth inhibition and trigger a mechanism of programmed cell death bearing the hallmarks of apoptosis (Casotti et al. 2005). This result indicates that PUAs may function not only as chemical defenses against grazers, but also as signalling molecules within diatom populations.

The hypothesis that PUAs may function as signalling molecules implies that mechanisms other than crushing of the cell by grazers can trigger their production and their release into the water. Freshwater chrysophytes produce PUAs, the same as those isolated from diatoms, which confer a rancid smell to the grazer-free growth medium

(Watson and Satchwill 2003), indicating, therefore, that these compounds can be produced and released in the water independently from herbivore attack. Cell lysis caused by osmotic stress has been shown *in vivo* to trigger PUA production in both freshwater chrysophytes (Satchwill et al. 2007) and diatoms (Wendel and Juttner 1996). This clearly indicates that any factor that triggers cell lysis can potentially induce PUA production and their release in the water. The process of lysis in phytoplankton has been shown to be exogenously triggered, e. g. bacterial or viral attack, nutrient limitation (Mitsutani et al. 1992; Berges and Falkowski 1998, Bidle and Falkowski 2004) or endogenous, e .g. genetically-based cell death (for review Franklin et al. 2006). Lysis appears to be a widespread phenomenon occurring at sea (Kirchman 1999) and high lysis rates have been shown as strongly occurring during final stages of blooms (Van Boekel et al. 1992; Kim et al. 1998). Only few estimates of diatom cell lysis rates during blooms are available as yet, and the importance of cell lysis for the dynamics of diatom populations and the release of organic compounds is apparently highly dependent on environmental factors (Van Boekel et al. 1992; Brussaard et al. 1996; Casotti et al. 2004). Chemical detection of dissolved PUAs is still in its infancy (Georg Pohnert, pers. comm.) but this is expected to represent a key step to allow further investigation of the ecological role of these compounds in marine ecosystem.

If significant amounts of dissolved PUAs can be detected in seawater, the question arises as to what are their effects to nearby organisms? One possibility is that PUAs act as allelochemicals against phytoplankton and bacteria. The toxicity of decadienal

has been demonstrated on non-marine bacteria (Bisignano et al. 2001, Adolph et al. 2004) and on the diatom *Thalassosira weissflogii* (Casotti et al. 2005), supporting this hypothesis. Another possibility is that PUAs may act as infochemicals modulating population density and/or indicating the presence of pathogens or predators, as it has been suggested for freshwater chrysophytes (Watson 2003). Alternatives roles of PUAs have been proposed in other organisms, such as decadienal and decatrienal used as an attractant in a bait-type insecticide against the corn rootworm (Coleoptera) (Hibbard et al. 1997), as a sexual hormone in the marine insect *Trochpus plumbeus* (Cheng and Roussis 1998), and heptadienal as pheromone in the swallowtail butterfly *Papilio machaon* (Omura et al. 2001). Therefore, the investigation of diatom-derived PUAs as chemical tracers, signaling or mediating changes in algal community biomass, structure and dynamics, in response to exogenous and endogenous triggers, becomes of great interest.

IV. Aims of the thesis

The aims of this thesis were to investigate:

1) The modulation of PUA production in cultured diatoms by different ecological factors.

After testing different methods of quantification and optimizing the protocol, PUA production was determined during the growth of diatom cultures under standard growth conditions, different light conditions and UV exposures, salinity gradients, different levels of nutrient limitation and in the presence of co-occurring copepods. Analyses of precursor PUFAs and PUAs were performed in collaboration with Thomas Wichard (University of Princeton, USA), Charles Vidoudez and Georg Pohnert (Schiller University of Jena, DE).

2) The putative role of PUAs as allelochemicals.

This hypothesis was tested by inoculating cultures of algae belonging to different taxonomical groups to a range of aldehyde concentrations. Growth, but also viability and cell membrane integrity were obtained using flow cytometry in order to assess and quantify their effect. The effect of PUAs was also tested on cultures of marine bacteria, including some strains isolated during PUA-producing diatom blooms, in collaboration with Laurent Intertaglia and Philippe Lebaron from the Observatoire Océanologique de Banyuls (France) within the framework of the RMP MARPLAN, which is a Research Mode Project supported by the European Network of Excellence MARBEF (Marine Biodiversity and Ecosystem Functioning).

3) Lysis rates at sea during diatom blooms.

This part was divided into field and laboratory components. The field component was performed to estimate lysis rates during PUA-producing diatom blooms that took place in the Northern Adriatic Sea in March 2002, 2004, 2005 and 2006 during the late winter bloom of *Skeletonema marinoi*. Similar experiments were also conducted in May-June 2003, 2004, within the framework of an interdisciplinary collaborative project focused on the biological oceanography of Northern Adriatic Sea (INTERREG Italy-Slovenia Project). Laboratory experiments were performed with *Skeletonema marinoi* isolated from this study area to test and optimize the different methods of lysis rate measurement.

Aldehyde production of *Skeletonema marinoi* in culture

I. Methodological aspects – Optimization of PUA extraction

I.1. Introduction

Two different methods have been reported in the literature to extract polyunsaturated aldehydes (PUAs) from freshwater diatoms: by direct headspace extraction (Wendel and Juttner 1996) and solid phase microextraction (SPME) (Pohnert 2000; Spiteller and Spiteller 2000). Since PUAs are particularly reactive and volatile, they are now preferentially extracted by procedures based on the transformation of PUAs into products that are easier to handle, such as ethyl ester by Wittig reaction with carbetoxyethylidene-triphenylphosphorane (CET-TPP) (d'Ippolito et al. 2002a, 2002b, 2003), and pentafluorobenzyl-oxime derivative by *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA·HCl) (Wichard et al. 2005b).

In order to test which method for PUA quantification was the best suited for physiological experiments, the results obtained using both methods were compared. Axenic triplicate cultures of the marine diatom *Skeletonema marinoi* (Sarno and

Zingone), strain CCMP 2092, were grown under standard growth conditions (see section II for details). Cell concentrations were monitored daily by flow cytometry (FACScalibur, Becton Dickinson), to determine the growth phase of the culture. Samples were taken in mid-exponential, early stationary and early declining phase of growth (see section II).

The determination of potential PUA production was first performed according to the method developed by d'Ippolito et al (2003). Briefly, a cell pellet (3 g wet weight) was obtained by centrifugation (1200g, 10 minutes) in order to collect approximately 10^8 cells. The cell pellet was resuspended in distilled water (1 mL) and sonicated for 1 min at 4° C. After 30 min, acetone (1 mL) was added and the resulting suspension sonicated again for 1 min and centrifuged at 2000g for 5 min. The acetone extraction was repeated three times. The supernatant was transferred to a separation funnel and extracted twice with CH_2Cl_2 (about 6 ml of solvent). The organic layers were combined, dried over dry Na_2SO_4 and then evaporated at reduced pressure with a rotary evaporator (Buchi, Rotavapor R-114). The oily extract was dissolved in CH_2Cl_2 (1 mL) and treated with the derivatizing reagent CET-TPP. The reaction mix was stirred at room temperature for 15-18 h and then the organic solvent was evaporated at reduced pressure. The residue was dissolved again in CH_2Cl_2 to a final concentration of 1 $\mu g\ \mu L^{-1}$ and directly analyzed by GC/MS at the Istituto di Biochimica Molecolare of Pozzuoli (IT) by Dr. Giuliana d'Ippolito.

PUAs were also measured according to Wichard et al. (2005). Cells were harvested in order to collect approximately 3 x 10^7 cells by gentle filtration and then

cells were resuspended in 2 mL of their original growth medium by carefully pipetting.

Different 25 mm filters (2 and 3 µm Nucleopore, 0.45 and 0.8 µm Millipore, 0.7 µm GF/F Whatman and 0.8 µm Versapore) were tested in order to obtain the maximum percentage of cell recovery. The percentage of recovery after filtration of 10^6 and 10^7 cells total varied over the six different filters (Figure II-1). Considering the two quantities of cells, the best filtration efficiency (maximum recovery with the smallest standard variation) was obtained with 0.8 µm Versapore filters, which therefore have been used afterwards.

The resulting cell suspensions were divided in four parts of equal volume. One subsample was used to determine cell concentrations by flow cytometry, in triplicate counts. The three additional subsamples were used for further chemical analyses to obtain additional technical replication for each replicate culture. Then 1 ml of the derivatizing agent *O*-2,3,4,5,6-pentafluorobenzyl hydroxylamine hydrochloride (25 mmol L^{-1} PFBHA in 100 mmol L^{-1} Tris-HCl, final pH 7.0) with 5 µl of an internal standard (1 mmol L^{-1} benzaldehyde in methanol, Fluka). Each subsample was sonicated for 1 min (Branson Sonifier 250) to disrupt cell integrity and to trigger PUA production. The cell fragments were incubated for 30 min at 17 °C, enough time to reach a stationary level of PUA (Pohnert 2000) and to allow for quantitative derivatisation (Wichard et al. 2005b). In this way, any PUA released by damaged cells immediately reacts with PFBHA and is derivatized.

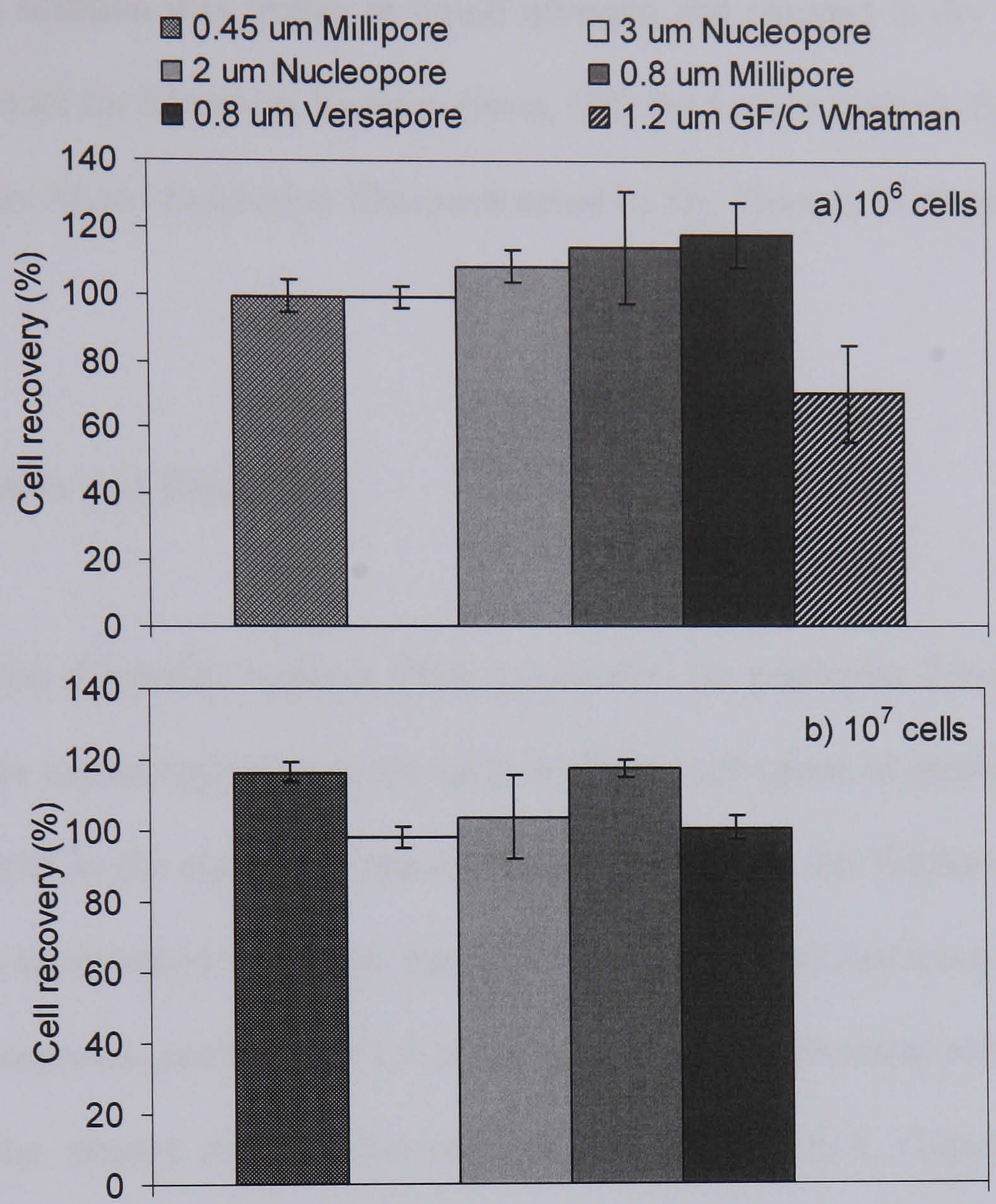


Figure II-1. Percentage cell recovery with (a) 10^6 cells and (b) 10^7 cells by using different filters. Data are means ± SD, n = 3 replicates from independent experiments. The 1.2 μm Whatman filter was not tested with 10^7 cells due to the low percentage cell recovery obtained with 10^6 cells.

Finally, the solution was frozen in liquid nitrogen and shipped in dry ice to the Max Planck Institute for Chemical Ecology (Jena, GR) for further analysis by GC/MS using a Solid Phase Micro-Extraction fiber performed by Dr. Thomas Wichard.

I.2. Results and Discussion

Using the d'Ippolito method, PUA production (in particular 2,4-octatrienal) was higher in the exponential than in the early declining cell phase of growth and no PUAs were detected in the stationary phase (Figure II-2). With the Wichard method, PUA production appeared to be higher during the stationary and declining cell phase than during exponential growth, with 2,4-heptadienal the most abundant aldehyde produced all over the phases and 2,4,7-octatrienal the minor PUA (Figure II-2). These contradictory results obtained with the two methods may be due to a lower sensitivity of the d'Ippolito method, as evidenced by the no PUA detected in the early stationary phase sample. Using the d'Ippolito method would imply the use of higher cell numbers ($>10^8$) in order to avoid the problem of detection limit, and, in our case, this required the cultivation of 10 L carboys of culture. As a consequence of the large volumes needed, the procedure is very time consuming and the manipulation of the samples adds a bias to the results.

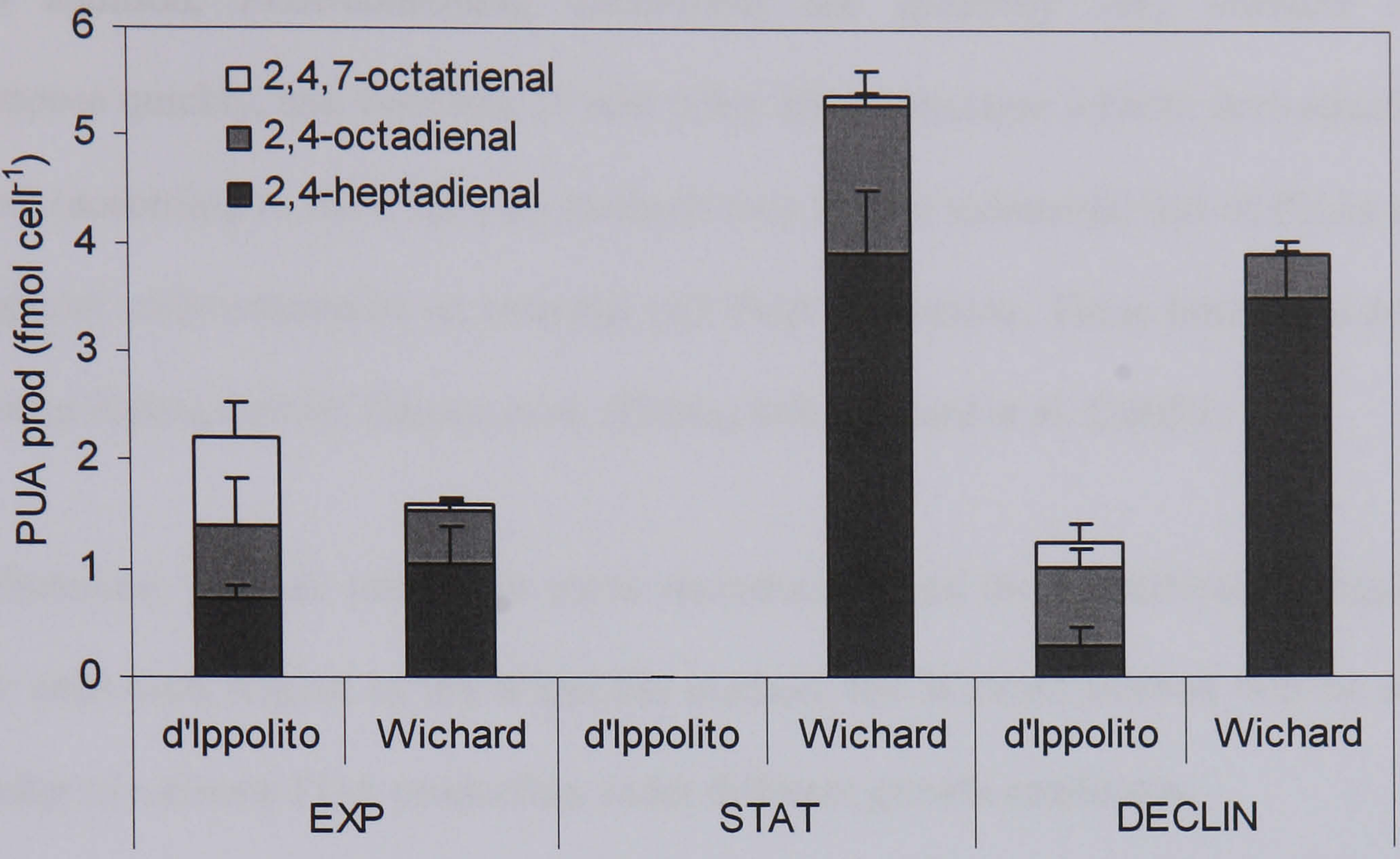


Figure II-2. Comparison of d'Ippolito's and Wichard's method for the determination of PUA production at the exponential (EXP), early stationary (STAT) and early declining phase (DECLIN). Different PUAs are indicated in the legend. Data are means of replicates from 3 independent experiments. Error bars are standard deviations.

In addition, PUA-containing compounds are generally very unstable and decompose quickly, and therefore 30 min delay after sonication without derivatization reagent (according to the d'Ippolito method) may lead to substantial lost of PUAs and subsequent underestimation of potential cell PUA production. These limitations have also been highlighted by Hansen et al. (2004a) and Wichard et al. (2005b).

Therefore, because results are more reproducible and the quantification requires fewer cells with respect to the d'Ippolito method, the Wichard method will be used hereafter to estimate PUA production under different growth conditions.

II. Factors influencing PUA production in *Skeletonema marinoi*

(Results on the modulation of PUA production during the growth cycle and under N- and P-limitation in batch cultures are published in Ribalet, F., Wichard, T., Pohnert, G., Ianora, A., Miralto, A., Casotti, R., 2007. Age and nutrient limitation enhance polyunsaturated aldehyde production in marine diatoms. Phytochemistry 68, 2059-2067).

II.1. Introduction

The diatom *Skeletonema marinoi* (separated from *S. costatum*, Sarno et al. 2005) forms dense, almost monospecific blooms in late-winter in the Northern Adriatic Sea (Mediterranean Sea) that strongly affect copepod reproduction and recruitment (Miralto et al. 1999; Ianora et al. 2004). This diatom produces the C_7 aldehyde 2E,4E/Z- heptadienal (in the following 2,4-heptadienal), as well as the C_8 aldehydes 2E-4E/Z-octadienal (2,4-octadienal) and 2E,4E/Z,7Z-octatrienal (2,4,7-octatrienal), which are derived from eicosapentaenoic (EPA), hexadecatrienoic (HDTRI), and hexadecatetraenoic (HDTETRA) acid, respectively (d'Ippolito et al. 2004).

PUAs are not present in intact cells and are synthesized mainly by wounded cells (Pohnert 2000). This reaction is under the control of a phospho-galactolipase/lipoxygenase/hydroperoxide lyase enzyme cascade (Pohnert 2002; d'Ippolito et al. 2004) which is activated within seconds after crushing of the cell. As

the transformation occurs immediately after cell disruption, the regulation through transcription and *de novo* protein biosynthesis of the enzymes is highly unlikely. A strict correlation has been found between PUA and PUFA composition of phospholipids and chloroplast-derived glycolipids in diatoms (Pohnert 2002; d'Ippolito et al. 2004). Since virtually no free PUFAs have been found in intact cells, and addition of PUFAs to wounded diatoms leads to increased formation of PUAs, availability of free fatty acids appears to be a limiting factor for aldehyde production (Pohnert 2002).

It is unclear how PUA production varies depending on the physiological conditions before cell disruption occurs. Variability in cell lipid content has been observed in response to changes in physiological conditions or environmental factors, including age of culture, nutrients or UV exposure (see for review Groth-Nard and Robert 1993; Skerratt et al. 1998). For many phytoplankton species, production of toxins and other secondary metabolites is strongly modulated by physiological conditions and age (reviewed in Legrand et al. 2003; Ianora et al. 2006). For instance, domoic acid is produced in higher quantities by the diatom *Pseudo-nitzschia* spp. during the stationary growth phase and under silica and phosphorus limitation, but not under nitrogen limitation since domoic acid contains nitrogen (Pan et al. 1998). Other stresses can also modulate toxin production in diatoms, such as high light and UV exposures that have been shown to increase the production of domoic acid (see Bates et al. 1998) and dimethylsulfoniopropionate (Sunda et al. 2002). In addition, the production of paralytic shellfish toxin in the marine dinoflagellates *Alexandrium*

minutum increases in the presence of copepods (Selander et al. 2006) and with increasing salinity (Grzebyk et al. 2003).

The modulation of toxin production during the growth cycle and with environmental conditions have important implications for ecophysiological studies since in most of the laboratory experiments, exponentially growing cultures are used to test for possible toxic effect on grazers, and could therefore lead to underestimate the impact of toxin-producing microalgae in nature.

The present chapter aims at quantifying potential production of PUAs per cell in cultures of *S. marinoi* during the different growth phases, from exponential to declining. In addition, PUA production has been measured also in cultures grown under different light and UV exposures, salinity gradients, different nutrient conditions (N-, P- and Si-limitation) and in the presence of co-occurring copepods. The data obtained show that the wound-activated production of PUAs per cell of *S. marinoi* is higher when cultures reach the stationary growth phase and that nutrient concentrations strongly modulate PUA production.

II.2. Material and method

II.2.1 Biological material

An axenic culture of the marine diatom *Skeletonema marinoi* Sarno and Zingone, strain CCMP 2092 (separated from *S. costatum*) from the Northern Adriatic Sea was

obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Boothbay Harbor, USA). Axenicity was confirmed at the start and the end of every experiment on any replicate by inoculating 1 mL of culture in 0.1% peptone agar in medium (in case of contamination, samples were not analyzed). One-month-old natural seawater amended with f/2 nutrients (Guillard 1975) was used as the medium. The cultures were maintained in a growth chamber (Hereaeus) at 17 °C on a 12h-12h light-dark cycle under a photon flux density of 150 μmol quanta m^{-2} s^{-1} provided by white fluorescent tubes (Phillips TLD 36W/950). Cell concentrations were monitored daily by a flow cytometer (FACScalibur, Becton Dickinson), equipped with standard optics (Casotti et al. 2005). Growth rates were calculated as μ (d^{-1}) according to:

$$\mu(\mathrm{d}^{-1}) = \ln \frac{N_1 / N_0}{t} \qquad (1)$$

where N_0 and N_1 represent cell concentration at the start and the end of the growth period, and t the time considered (usually 1 d).

II.2.2 Experimental set-up

(1) Growth phase

To investigate the production of PUAs per cell during growth in culture under standard conditions, several independent batch cultures were sampled in mid-

exponential (constant maximal growth rate), early stationary (as soon as the growth rate decreases) and declining phases of growth (after a substantial decrease in cell concentration). These phases have been chosen since they represent critical steps in the evolution of an algal culture, being characterized by different dominant cell processes (growth, senescence and mortality) that are known to affect cell metabolism. Samples were always taken 6 h after the onset of the light period in order to avoid interference from circadian variability.

(2) Macronutrient

Two different approaches were used to assess the effect of nutrient limitation on PUA production.

a) Duplicate cultures were grown continuously in cyclostats (chemostats with light:dark cycle) in f/2 medium with a N:P:Si ratio of 24.5:1:3 (NO_3^- = 883 μmol L^{-1}, PO_4^{3-} = 36 μmol L^{-1}, $Si(OH)_4$ = 107 μmol L^{-1}, considered to be nutrient-replete), 4:1:3 (NO_3^- = 45 μmol L^{-1}, PO_4^{3-} = 36 μmol L^{-1}, $Si(OH)_4$ = 107 μmol L^{-1}, considered to be nitrogen-limited), 80:1:3 (NO_3^- = 883 μmol L^{-1}, PO_4^{3-} = 11 μmol L^{-1}, $Si(OH)_4$ = 107 μmol L^{-1}, considered to be phosphorus-limited) and 24.5:1:1 (NO_3^- = 883 μmol L^{-1}, PO_4^{3-} = 36 μmol L^{-1}, $Si(OH)_4$ = 36 μmol L^{-1}, considered to be silicon-limited). Cultures (500 mL) were grown in 1-L polycarbonate bottles for at least 6 generations, allowing cells to acclimate to the nutrient-limited conditions. The cyclostat apparatus (i. e. chemostat on a light-dark cycle) is shown in Figure II-3. The culture was stirred by suspended magnetic stirrer and sterile air was bubbled into the chamber near the bottom.

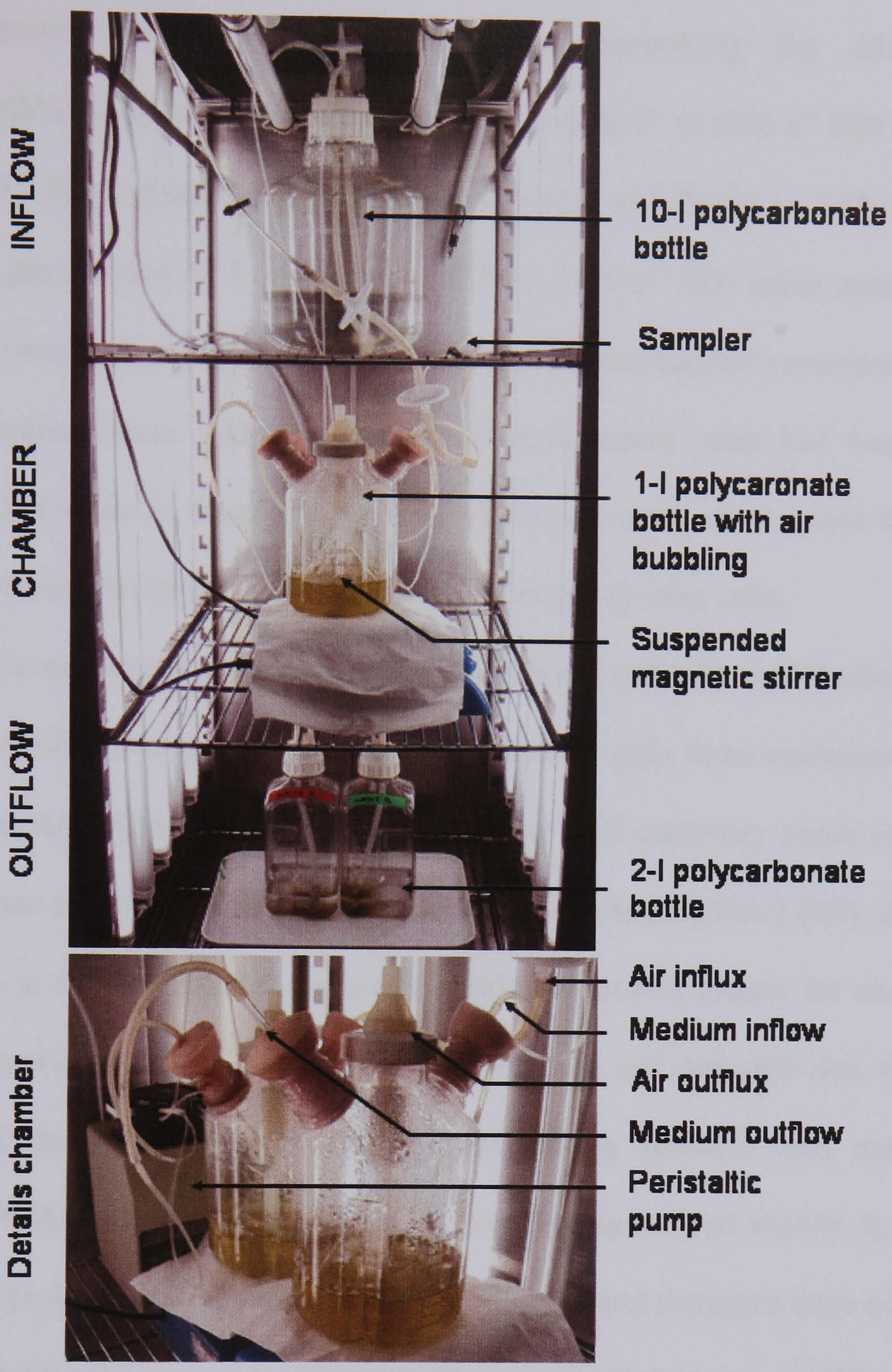


Figure II-3: Cyclostat apparatus used in the study. The cyclostat chamber was a 1-L polycarbonate bottle with three tubes for air influx, medium inflow and overflow. The bottle tap allows air outflow. The chamber was stirred vigorously by suspended magnetic stirrer and sterile air was bubbled into the chamber near the bottom.

The growth rates were manipulated by controlling the dilution rates (inflow/outflow of the medium) and were set up at 0.90 d^{-1} (± 0.06 d^{-1} SD) and 0. 28 d^{-1} (± 0.03 d^{-1} SD), representing 93.7% (± 6.2 %SD) and 29.2% (± 3.1% SD) of the maximum growth rate of *S. marinoi* (0.96 d^{-1} ± 0.05 d^{-1} SD) under nutrient-replete conditions (see above). Daily samples for cell counts and nutrient concentrations in the outflow medium were taken to determine when steady state had been reached. Samples were taken for chemical and flow cytometric analyses to assess the effect of different nutrient limitation on PUA production in steady state cells.

b) Triplicate cultures were grown in batch cultures under N- or P-limited media (as described above), inoculated with N- and P- limited cells from cyclostats (set-up at low dilution rate). Cultures were allowed to grow until stationary phase of growth in polycarbonate flasks with air bubbling. Growth rate was monitored daily and samples were taken at the exponential and early stationary growth phases for chemical and flow cytometric analyses. The declining phase was not sampled due to the high fragility of dying cells which easily broke during filtration and manipulation, liberating PUAs before addition of the derivatizing agent (see section I). Si-limited batch cultures did not show a clear exponential phase and therefore were not sampled. This growth bioassay aimed to determine PUA production in pre-conditioned cells to nutrient limitation.

(3) Light

To assess the effect of different photon flux densities on PUA production, triplicate cultures were grown at a flux of photosynthetically active radiation of 10 μmol quanta

m^{-2} s^{-1} (LOW); 150 µmol quanta m^{-2} s^{-1} (STANDARD); and 450 µmol quanta m^{-2} s^{-1} (HIGH). All cultures were grown with air bubbling in fully nutrient-replete f/2 medium. Triplicates cultures were grown semi-continuously (i. e. by serial dilution) for at least 6 generations, allowing cells to acclimate to the different photon flux densities. Samples were taken in mid-exponential, early stationary and early declining phases of growth.

(4) UVB

To investigate the effect of UV radiation on PUA production, a 2-L exponentially-growing culture was split into four and two replicates were transferred to two sterile 1-L quartz bottles (Trallero and Schlee, Spain), which are transparent to UV radiation, while two replicates were kept in polycarbonate bottles, which are usually used to grow the algae and which filter the UV light out. The filtration of UVA and UVB radiations was checked for each bottle using a spectrophotometer (Hewlett Packard 8453 Spectroscopy System). All cultures were grown with air bubbling in f/2 medium under a flux density of photosynthetically active radiation of 150 µmol quanta m^{-2} s^{-1}. UVB was supplemented 4 h after the onset of the light period during 4 h (13:00 – 17:00) by 40 W fluorescent lamps (Philips TL 40 W/12) at two levels: 0.05 W UVB m^{-2} $s^{-}$and 0.15 W UVB m^{-2} s^{-1}. A Dr. Gröbel RM-11 (UV-Elektronik) broadband radiometer equipped with sensors for UVA and UVB was used to measure UV flux in the different cultures. Samples were taken before UV exposure (t 0h), after UV exposure (t 4h) and 20 h later (t 24h).

(5) Salinity

In order to assess whether PUA production differs along a salinity range, cells were grown in media with different salinities obtained by mixing seawater from the Bay of Naples with distilled water, amended with f/2 nutrients (Guillard 1975) and then autoclaved. Salinity was measured after autoclaving by a conductivity meter (30M-Yellow Spring Inc.). Cultures were pre-acclimated to the lower salinities by transferring aliquots of cultures to media with a 2.5 psu lower salinity than the previous one every third day. In cases where rapid decrease in growth was observed over a 2.5 psu-decrement, strains were transferred to salinity only 1 or 1.5 psu lower than the one in which growth was still observed. Experiments were performed at salinities of 26 and 16 psu. Cultures (1 L) were grown in 2-L polycarbonate bottles for at least 6 generations, allowing cells to acclimate to the salinity. Cultures grown at salinity of 36.5 psu (seawater taken from the Bay of Naples, IT), were used as control.

(6) Presence of copepods

In order to assess whether the presence of copepods influences the PUA production, 10-L cultures were grown exponentially with 50 *Calanus helgolandicus*, isolated from the Northern Adriatic Sea during the bloom of *Skeletonema marinoi*. Nutrient concentrations and light conditions were those used under the standard growth conditions (see above). In the first experiment, copepods were pre-fed for three days on the dinoflagellate *Prorocentrum minimum* diet and then carefully washed (to remove all phytoplankton cells) and inoculated in the diatom culture. In the second experiment, copepods were starved for three days and then put in 50-mL Falcon tubes

equipped with 0.22 μm Steriflip filters (Millipore) filled filtered medium of the culture, at a concentration of 10 copepods per tube. In the third experiment, copepods were pre-fed on *S. marinoi* diet for three days and then washed and inoculated in a pre-washed dialysis tubing cellulose membrane (Sigma Aldrich D9402) filled with filtered medium of the culture. Samples were taken at t 0h, 24h, 48h and 72h after copepod inoculation. For each experiment, 10-L cultures of *S. marinoi* were used as control.

II.2.3 Cell properties and viability

A Becton-Dickinson FACScalibur flow cytometer equipped with an air-cooled 488 nm argon-ion laser at 15 mW power was used. The sheath fluid was natural seawater filtered onto 0.22 μm polycarbonate filters (Nuclepore), salinity of the sheath fluid was adjusted for the experiment with lower salinities. Both the sheath fluid and sample velocities (40 μL min^{-1}) were kept constant during all of the analyses. Optical properties of cells, as measured by flow cytometry in different culture conditions, were compared using forward angle light scatter (FALS) and right angle light scatter (RALS). Red fluorescence (RED) was collected through a 650 long-pass filter and was also used as a proxy for chlorophyll cell content. All values were expressed as units relative to the beads used as internal standards (3.7 μm Coulter FlowSet Fluorospheres). Data acquisition (10^4 cells on average for each sample) and analysis were performed using CellQuest software (Becton-Dickinson). Counts were triggered on RED signals, with a threshold of 52.

Percentages of viable cells were assessed using the vital stain SYTOX Green (Molecular Probes) using flow cytometry (Casotti et al. 2005). This stain does not penetrate live cells but only those with compromised plasma membranes. Optimal final concentration (500 nmol L^{-1}) and time of incubation (10 min) were assessed experimentally. The green fluorescence of stained cells was collected through a 530/30-nm bandpass filter. Only SYTOX Green-negative cells were considered for calculation of PUA content, as it was assumed that inactive cells would not be able to activate processes leading to PUA production.

II.2.4 Analysis of PUAs

The quantification of PUAs was assessed following a slightly modified protocol as described by Wichard et al. (2005b) (see section I). PUA extraction was performed by Dr. Thomas Wichard (Max Plank Institute for Chemical Analysis, Jena, DE). The limit of quantification was 5 ng mL^{-1} and the coefficient of variation between subsamples originating from the same replicates averaged 12%. The means of the technical replicates were used for the determination of the standard deviation for repeated measurements with the three independent cultures.

II.2.5 Analyses of PUFAs

Quantification of the polyunsaturated fatty acids (PUFAs) which have been identified as PUA-precursors in *S. marinoi*: eicosapentaenoic (20:5 n-3, EPA), hexadecatrienoic (16:3 n-4, HDTRI), and hexadecatetraenoic acid (16:4 n-1, HDTETRA), was performed after trans-esterification following the procedure of Rodriguez-Ruiz et al. (1998) For replication, three independent cultures were filtered each onto separate GF/F filters (Whatman), frozen in liquid nitrogen and stored at -80 °C until further analysis by GC/MS carried out by Dr. Thomas Wichard (Max Planck Institute for Chemical Analysis, Jena, GR). The PUFAs were identified by comparison with synthetic (HDTETRA, Pohnert et al. 2004), or commercially available standards (EPA). Hexadecatrienoic acid was identified from its mass spectrum according to Dodds et al. (2005).

II.2.6 Analyses of particulate nitrogen, phosphorus and silic acid

Samples for particulate C and N (POC and PON, respectively) from each independent culture were filtered on acidified pre-combusted (450 °C, 24 h) GF/F filters and stored at -80 °C until analysis by CHN elemental analyzer (Perkin Elmer 2400) performed by Dr. Daniele Cassin (CNR-Istituto di Scienze del Mare of Venice, IT). Organic phosphorus (POP) was measured colorimetrically after decomposition in 1% potassium persulfate solution for 1 h at 121°C (Parsons et al. 1984a). Samples for

silicic acid content were filtered through polycarbonate filters of 0.8 µm pore size, using a plastic filtering apparatus. Biogenic silica was measured colorimetrically after hydrolysis by 0.5% Na_2CO_3 solution for 2 h at 85°C and neutralization of the contents with 0.5 N HCl (Paasche 1980). POP and particulate Si analysis was performed with the help of Federico Corato and Rosario Lavezza (Stazione Zoologica di Napoli) and Francesco Acri (CNR-Istituto di Scienze del Mare of Venice, IT) using an autoanalyzer (Easychem Plus, Systea Srl).

II.2.7 Analyses of chlorophyll a

Between 15 and 30 mL of cultures, depending on cell concentrations, were filtered through 25-mm GF/F filters (Whatman) and immediately frozen in liquid nitrogen. For the experiments performed under standard growth conditions and under nutrient limited batch culture, frozen filters were mechanically ground in 100% methanol and the extract injected into a HP 1100 system HPLC as outlined in Casotti et al. (2005). Analysis of Chl *a* concentrations were performed by Dr. Christophe Brunet (Stazione Zoologica di Napoli, IT). For the other experiments, Chl *a* concentrations were measured spectrophotometrically after extraction in 90% acetone for 2 h at room temperature followed by 20 min centrifugation at 3000g (Jeffrey and Humphrey 1975).

Chl *a* concentrations were calculated as:

$$\text{Chl } a\ (\mu g\ L^{-1}) = \frac{V_{extract}}{V_{filtered}} \times (11.47\ \lambda_{664} - 0.4\ \lambda_{630}) \qquad (2)$$

Where λ_{664} and λ_{630} represent the absorption at 664 nm (for Chl *a* peak) and 630 nm (for Chl *c* peak), respectively, and $V_{extract}$ and $V_{filtered}$ are the volume of extract and the volume filtered (in mL) (Jeffrey and Humphrey 1975).

II.2.8 Alkaline phosphatase measurement

Alkaline phosphatase activity was assayed on continuous cultures grown in P-limited medium by using the fluorogenic substrate methyl-umbelliferyl-phosphate (Sigma Aldrich) (Fernley and Walker 1965) according to Perry (1972). Substrate solution was added to 3-ml water samples at a final concentration of 36 μmol L^{-1}. Initial kinetic study indicates that this concentration is saturating (data not shown). Samples were incubated at room temperature, then subsamples were removed and assayed immediately at intervals adjusted to the activity of the sample, such that all readings fell within the linear range of the assay (at least 5 measurements within 2 h or less). The methylumbelliferon produced was detected as increase in fluorescence (excitation at 360 nm and emission at 460 nm) by a spectrofluorometer (CaryEclipse, Varian). A standard curve from 0 to 8.5 μmol L^{-1} methylumbelliferon (Sigma Aldrich) was used to calculate the rate of methylumbelliferon-phosphate hydrolysis.

II.3. Results

II.3.1 PUA production under standard growth conditions

The average growth rate of *S. marinoi* in exponential phase under standard growth conditions was 0.96 d^{-1} ± 0.05 d^{-1} SD. The cultures attained cell concentrations of 1.05 10^6 cell ml^{-1} at the stationary phase, and these remained constant for 6 days before the declining phase (Figure II-4a). The hypothesis that nitrate, phosphate or silicon were the factors limiting growth in the control cultures causing their entrance in the stationary phase of growth was tested by adding these nutrients separately to triplicate control cultures in the same concentrations as in the f/2 medium (883, 36 and 107 µmol l^{-1}, respectively) at the onset of the stationary phase of growth. The cultures showed an increase in cell concentration with silicon addition, indicating that Si depletion triggered the onset of the stationary phase, while nitrogen and phosphorus could be excluded as factors limiting growth (data not shown).

No significant change in Forward Angle Light Scatter (FALS, used as a proxy of size) was observed (ANOVA, $p>0.05$), while Right Angle Light Scatter (RALS, indicator of internal granular structure) increased progressively with time (Table II-1). The increase in RALS was paralleled by an increase in granularity of the diatom cytoplasm, as observed by microscopy. Red fluorescence from chlorophyll increased by 47.1% from the exponential to stationary phase and then decreased again during the declining phase (Table II-1). Chl *a* from HPLC followed the same trend (Table II-1).

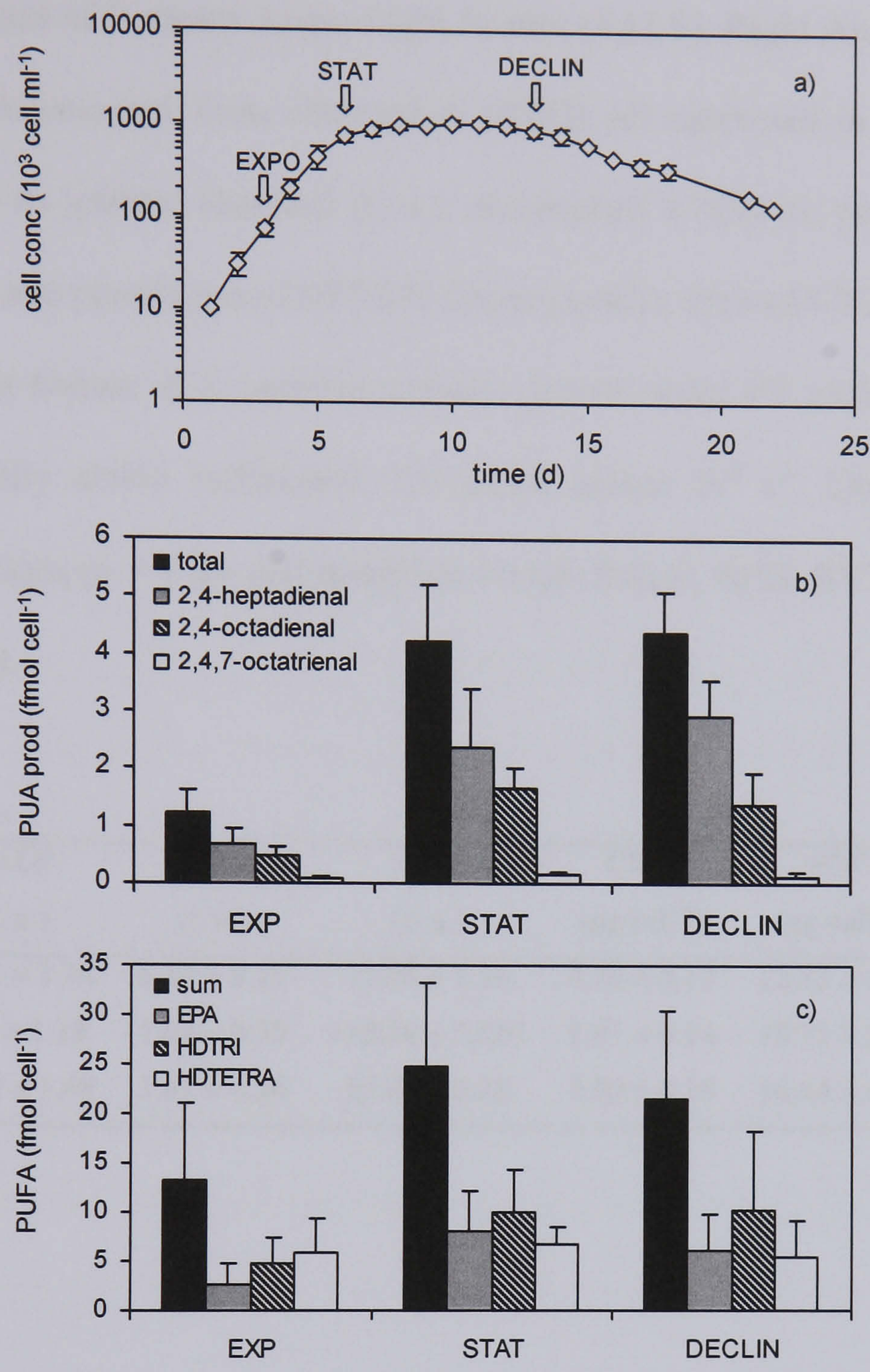


Figure II-4. (a) Average cell concentrations of *S. marinoi* during growth in batch cultures grown under standard growth conditions (see Method). Arrows indicate sampling points for analyses of polyunsaturated aldehydes (PUAs) and their precursor polyunsaturated fatty acids (PUFAs). (b) PUA production in nutrient-replete cultures at the three growth phases indicated in panel (a). Different PUAs are indicated in the legend. (c) PUFAs at the three growth phases indicated in panels (a) and (b). Different PUFAs are indicated in the legend. Sum indicates the sum of the three PUA-precursor fatty acids. EPA is eicosapentaenoic acid, HDTRI is hexadecatrienoic acid, and HDTETRA is hexadecatetraenoic acid. For plots of nutrient-replete cultures, data are means of replicates from 9 independent experiments, while for nutrient-limited cultures, n = 3. EXP, STAT, and DECLIN indicate the exponential, stationary, and declining phase of growth, respectively. Error bars are standard deviations (when not visible, error bars are smaller than the symbol of the average cell concentration).

Table II-1. Values of Forward Angle Light Scatter (FALS), Right Angle Light Scatter (RALS), red fluorescence from chlorophyll (RED), all expressed in units relative to the beads used as internal standard (r. u.), chlorophyll a (Chl a), particulate organic carbon (POC), and percentage of SYTOX Green positive cells (SYTOX+, non-viable) during different phases of *S. marinoi* cultures grown under f/2 medium at a flux of photosynthetically active radiationof 150 μmol quanta m^{-2} s^{-1}. Data are means ± standard deviations (n = 9 for cell densities, FALS, RALS, RED, SYTOX+ and POC; n = 4 for Chl *a*).

Growth phase	FALS (r. u.)	RALS (r. u.)	RED (r. u.)	Chl *a* (pg $cell^{-1}$)	POC (pg $cell^{-1}$)	SYTOX+ (%)
EXP	10.53 ± 1.74	0.83 ± 0.15	79.04 ± 5.16	0.76 ± 0.17	12.85 ± 1.35	0.7 ± 0.1
STAT	9.50 ± 1.38	1.09 ± 0.30	116.26 ± 12.67	1.01 ± 0.14	15.71 ± 2.17	3.6 ± 0.8
DECLIN	10.12 ± 1.69	1.61 ± 0.26	55.67 ± 6.25	0.80 ± 0.15	16.64 ± 4.13	9.3 ± 1.5

The proportion of non-viable cells, assessed using the SYTOX Green nucleic acid stain, was very low in the exponential phase (SYTOX Green-positive cells 0.7% ± 0.1% SD), while it increased with culture age (3.6% ± 0.8% SD in the stationary and 9.3% ± 1.5% SD in the declining phase - Table II-1). Cellular C increased with culture age (Table II-1).

Total PUA content per cell produced in response to wounding in the exponential phase of growth was 1.23 fmol $cell^{-1}$ ± 0.37 fmol $cell^{-1}$ SD and increased significantly in the early stationary phase (4.21 fmol $cell^{-1}$ ± 0.99 fmol $cell^{-1}$ SD, Tukey Multiple Comparison post-test, $p<0.001$), remaining stable until the early declining phase (4.39 fmol $cell^{-1}$ ± 0.69 fmol $cell^{-1}$ SD, Figure II-4b). The relative increase followed the same trend when PUAs were normalized by Chl *a* or POC (data not shown). Among the PUAs, heptadienal was always the most abundant, accounting for 53.7% of total PUAs in exponential, 56.8% in stationary, and 68.8% in declining phases of growth. Octadienal contributed 39.5% of total PUAs in exponential, 39.6% in stationary and 32.3% in declining phases (Figure II-4b). Octatrienal accounted for a lower proportion of total PUAs with 6.8% in exponential, 3.7% in stationary and 2.6% in declining phase. Absolute values were significantly different only for exponential *vs* stationary phase for all three aldehydes (Tukey Multiple Comparison post-test, $p<0.01$).

Average content per cell of the three PUFAs which serve as precursors of the unsaturated aldehydes followed the same pattern than that observed for PUAs. These PUFAs were found in concentrations of 13.1 fmol $cell^{-1}$ ± 7.9 fmol $cell^{-1}$ SD during the exponential phase, corresponding to 3.4 pg $cell^{-1}$ ± 2.1 pg $cell^{-1}$ SD. Their level

increased to 24.7 fmol cell^{-1} ± 8.5 fmol cell^{-1} SD in the stationary phase (Tukey Multiple Comparison post-test, $p< 0.05$) and remained stable until the declining phase of growth (21.6 fmol cell^{-1} ± 8.8 fmol cell^{-1} SD, Figure II-4c). During the exponential growth phase, the dominant PUFAs were hexadecatetraenoic and hexadecatrienoic acids (5.9 fmol cell^{-1} ± 3.4 fmol cell^{-1} SD and 4.7 fmol cell^{-1} ± 2.7 fmol cell^{-1} SD, respectively), accounting for 44.8% and 35.6% of the three PUA precursors, while eicosapentaenoic acid was present in lower amounts (2.6 fmol cell^{-1} ± 2.1 fmol cell^{-1} SD, i.e., 19.6% of the three PUA precursors). In the stationary phase of growth, cell content in terms of hexadecatrienoic and eicosapentaenoic acids increased in absolute values to 10.1 fmol cell^{-1} ± 4.3 fmol cell^{-1} SD and 8.0 fmol cell^{-1} ± 4.2 fmol cell^{-1} SD, respectively. The total percent contribution of these two PUFAs was 40.6% and 32.5%, respectively. In contrast, the absolute amount of hexadecatetraenoic acid remained nearly constant (6.7 fmol cell^{-1} ± 1.9 fmol cell^{-1} SD), but this resulted in a lower percent contribution (26.9%).

II.3.2 PUA production under nutrient limitation in continuous cultures

Continuous cultures are maintained in balanced growth under nutrient-limited conditions at a growth rate that is equal to the dilution rate. Consequently, a decreasing growth rate leads to an increased nutrient limitation, while dilution rates set up at the maximum growth rate (nutrient-replete growth) do not induce nutrient limitation (Macintyre and Cullen 2005). In our case, cultures acclimated to low growth rate

(29.2% maximum growth rate) are called "nutrient limited" cultures while those acclimated to high dilution rate (93.7% maximum growth rate) are called "nutrient-replete" cultures.

N- and P-limited cells had 23.9% (± 2.1% SD) and 29.7% (± 12.8% SD) lower FALS values, respectively, relative to the nutrient-replete cells. FALS decreased only slightly in Si-limited cells (Table II-2). RALS values decreased in N-limited and increased in P- and Si-limited cells. Red fluorescence values were 71.4% (± 1.2% SD) lower in N-limited cells and 61.6% (± 5.0% SD) higher in Si-limited cells, while no difference was observed in P-limited cells with respect to nutrient-replete cells (since only duplicate cultures were performed, data were not analyzed statistically). Chl *a* content decreased strongly in N-limited cells (from 1.12 ± 0.13 pg cell^{-1} SD to 0.15 ± 0.03 pg cell^{-1} SD) and increased in Si-limited cells (from 1.12 ± 0.27 pg cell^{-1} SD to 1.99 ± 0.66 pg cell^{-1} SD) while it did not vary in P-limited cells. Cell viability was not affected in N- and P-limited cells, while it increased in Si-limited cells (from 9.0 ± 0.9% SD to 20.9 ± 5.7% SD) (Table II-2).

Table II-2. Values of Forward Angle Light Scatter (FALS), Right Angle Light Scatter (RALS), red fluorescence from chlorophyll (RED), all expressed in units relative to the beads used as internal standard (r. u.), chlorophyll *a* (Chl *a*), percentage of SYTOX Green positive cells (SYTOX+, non-viable), and cell concentration (10^6 cell ml^{-1}) of *S. marinoi* grown continuously at high dilution rate (HIGH) and low dilution rate (LOW) under nitrate-limited (N-limited), phosphate-limited (P-limited), and silicon-limited (Si-limited) growth medium. Data are means ± standard deviations (n = 2).

Dilution Rate	Growth medium	FALS (r. u.)	RALS (r. u.)	RED (r. u.)	Chl *a* (pg $cell^{-1}$)	SYTOX+ (%)	Cell conc (10^6 cell ml^{-1})
HIGH	N-limited	8.43 ± 0.20	1.19 ± 0.05	67.46 ± 1.90	1.12 ± 0.13	3.4 ± 0.8	0.27 ± 0.02
HIGH	P-limited	8.39 ± 0.64	0.68 ± 0.02	63.45 ± 3.69	1.02 ± 0.14	6.3 ± 1.5	0.23 ± 0.03
HIGH	Si-limited	8.38 ± 0.07	0.38 ± 0.01	55.47 ± 0.80	1.12 ± 0.27	9.0 ± 0.9	0.20 ± 0.02
LOW	N-limited	6.48 ± 0.11	0.60 ± 0.01	19.27 ± 0.44	0.15 ± 0.03	3.1 ± 1.2	0.47 ± 0.06
LOW	P-limited	5.96 ± 1.51	1.93 ± 0.19	59.05 ± 3.22	0.80 ± 0.60	7.2 ± 2.2	0.58 ± 0.11
LOW	Si-limited	7.72 ± 0.05	0.61 ± 0.02	89.62 ± 1.80	1.99 ± 0.66	20.9 ± 5.7	0.44 ± 0.03

Since alkaline phosphatase is regulated by phosphate supply, its activity increases when dissolved inorganic phosphate drops below critical level, and therefore is commonly used to infer P stress or P limitation (Hoppe 2003). The alkaline phosphatase activity in cultures grown in the P-limited medium reached 6.17 (± 0.32 SD) μmol P L^{-1} h^{-1} in cultures acclimated to low dilution rate while it remained very low (0.40 ± 0.03 SD μmol P L^{-1} h^{-1}) in those acclimated to high dilution rate (Table II-3). This result confirms therefore that low dilution rates induce P-limitation in continuous cultures while those acclimated to high dilution were not experiencing P-limitation.

The cell quota of every limited culture was related to the steady-state dilution rate as proposed by Droop (1974). Particulate Organic Carbon (POC), PON and POP per cell and the C:N, N:P and Si:N ratio were strongly affected in N- and P-limited cells (Table II-3). The important trends can be summarized as follows: (1) PON and POP per cell in both N- and P-limited cultures decreased while Si content was not clearly affected. (2) The N:P ratio decreased in N-limited cells and increased in P-limited cells, while the Si:N increased as a result of decreasing PON per cell in N- and P-limited cells.

Si-limitation induced very different responses on the cell chemical composition as compared to nitrogen and phosphorus, which are more closely linked to photosynthetic metabolism (Claquin et al. 2002). Si:N decreased as a result of decreasing Si content in Si-limited cells, while POC, PON and POP per cell were relatively constant, with mean values of 16.02 ± 2.02 pg $cell^{-1}$, 2.53 ± 0.28 pg $cell^{-1}$, and 0.58 ± 0.13 pg $cell^{-1}$, respectively (Table II-3).

Table II-3. Particulate organic nitrogen (PON), phosphate (POP), carbon (POC) and Biosilica (Si) cell content, molar C:N, N:P and Si:N ratios of *S. marinoi* cells grown continuously at high and low dilution rate in nitrate-limiting (N-limited), phosphate-limiting (P-limited), and silicon-limiting (Si-limited) growth medium. Alkaline phosphatase activity (APA) in cultures grown in P-limited medium is also indicated. Data are means ± standard deviations (n = 2)

		Growth medium		
		N-limited	P-limited	Si-limited
High Dilution Rate	PON (pg cell^{-1})	2.36 ± 0.43	2.51 ± 0.33	2.72 ± 0.23
	POP (pg cell^{-1})	0.47 ± 0.01	0.63 ± 0.13	0.50 ± 0.13
	POC (pg cell^{-1})	13.90 ± 0.81	14.73 ± 2.69	14.34 ± 0.65
	Si content (pg cell^{-1})	6.87 ± 1.24	6.58 ± 0.85	6.16 ± 0.77
	C:N	6.94 ± 0.87	6.83 ± 0.36	6.16 ± 0.25
	N:P	11.13 ± 2.24	9.18 ± 3.11	12.21 ± 2.07
	Si:N	1.45 ± 0.01	1.31 ± 0.01	1.13 ± 0.04
	APA (μmol P L^{-1} h^{-1})	-	0.40 ± 0.03	-
Low Dilution Rate	PON (pg cell^{-1})	0.77 ± 0.14	1.50 ± 0.06	2.34 ± 0.19
	POP (pg cell^{-1})	0.30 ± 0.01	0.29 ± 0.11	0.65 ± 0.10
	POC (pg cell^{-1})	8.90 ± 1.24	13.36 ± 1.05	17.69 ± 0.78
	Si content (pg cell^{-1})	8.25 ± 0.93	7.77 ± 0.75	3.10 ± 0.36
	C:N	12.64 ± 0.42	10.36 ± 0.37	8.85 ± 0.33
	N:P	5.73 ± 1.26	14.82 ± 4.67	7.99 ± 0.58
	Si:N	5.40 ± 0.38	2.57 ± 0.14	0.66 ± 0.02
	APA (μmol P L^{-1} h^{-1})	-	6.17 ± 0.32	-

Total PUAs produced per cell in the replete-nutrient cultures (high dilution rate) were similar under Si-limited (3.64 fmol $cell^{-1}$ ± 1.39 fmol $cell^{-1}$ SD), N-limited (3.22 fmol $cell^{-1}$ ± 0.03 fmol $cell^{-1}$ SD) and P-limited (2.61 fmol $cell^{-1}$ ± 0.77 fmol $cell^{-1}$ SD) growth media (Figure II-5a). Under nutrient limitation (low dilution rate), the production increased 7.5 times in Si-limited cells and 3 times in P-limited cells with PUAs production as high as 27.5 fmol $cell^{-1}$ ± 4.4 fmol $cell^{-1}$ SD in Si-limited and 8.53 fmol $cell^{-1}$ ± 2.6 fmol $cell^{-1}$ SD in the P-limited cultures (Figure II-5a). The increase in PUAs was mainly attributable to an increase in both heptadienal (13.9 fmol $cell^{-1}$ ± 2.1 fmol $cell^{-1}$ SD and 3.1 fmol $cell^{-1}$ ± 1.0 fmol $cell^{-1}$ SD in Si- and P-limited cells, respectively) and octadienal production (13.4 fmol $cell^{-1}$ ± 2.1 fmol $cell^{-1}$ SD and 5.2 fmol $cell^{-1}$ ± 1.5 fmol $cell^{-1}$ SD in Si- and P-limited cells, respectively) (Figure II-5b and c). In strong contrast, total PUA production decreased dramatically in N-limited cells and reached 0.14 fmol $cell^{-1}$ ± 0.1 fmol $cell^{-1}$ SD (Figure II-5b and c). Octatrienal was only present in traces in all cultures (Figure II-5b and c).

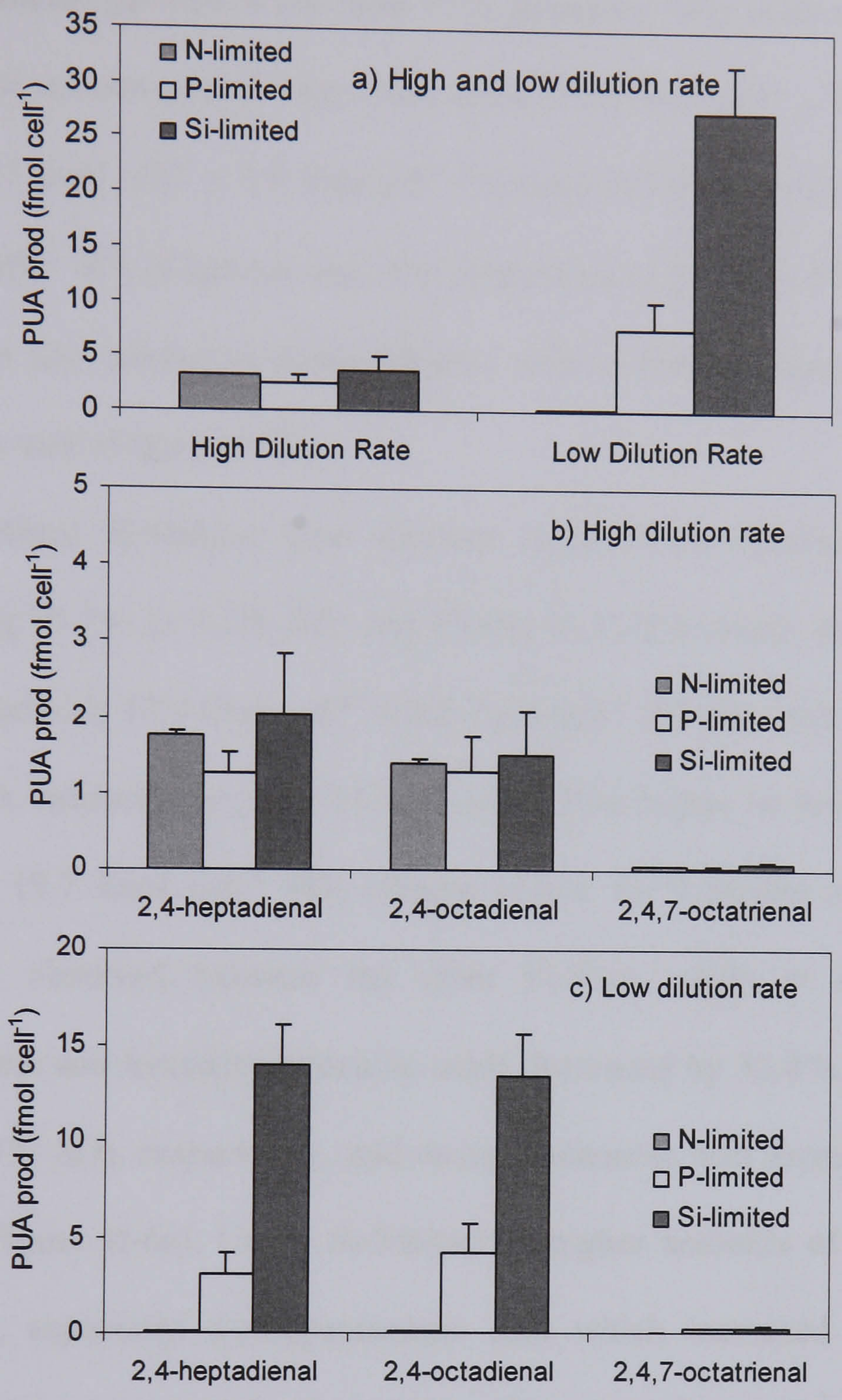


Figure II-5. (a) Total PUA production per cell in continuous cultures of *S. marinoi* acclimated at high and low dilution rate in nitrogen-limited (N-limited), phosphate-limited (P-limited), and silicon-limited (Si-limited) growth medium. (b) Composition in terms of different PUAs produced at high dilution rate, and (c) low dilution rate. Error bars are standard deviations. N = 2

Average content per cell of the three PUA precursor fatty acids was very similar in nutrient-replete cultures grown under Si-limited (19.8 fmol $cell^{-1}$ ± 1.4 fmol $cell^{-1}$ SD), N-limited (19.7 fmol $cell^{-1}$ ± 0.6 fmol $cell^{-1}$ SD) and P-limited medium (19.1 fmol $cell^{-1}$ ± 1.6 fmol $cell^{-1}$ SD) (Figure II-6a). The proportion of the three PUFA precursors of aldehydes was also similar in those cultures, with hexadecatetraenoic acid being the dominant fatty acid (Figure II-6b).

Under nutrient limitation (low dilution rate), PUFA content was differently affected, being 75.1% (± 3.1% SD) and 45.0% (± 11.8%) lower in N- and P-limited cultures, respectively (4.9 fmol $cell^{-1}$ ± 0.6 fmol $cell^{-1}$ SD and 10.5 fmol $cell^{-1}$ ± 2.26 fmol $cell^{-1}$ SD, respectively), and 337.1% (± 99.3%) higher in Si-limited cells (86.6 fmol $cell^{-1}$ ± 19.7 fmol $cell^{-1}$ SD) (Figure II-6c). In N-limited cultures, the same decrease was observed between the three PUFAs, while in P-limited-cultures, hexadecatrienoic and hexadecatetraenoic acids decreased by 32.8% (±17.3% SD) and 56.4% (± 16.1% SD), respectively, and eicosapentaenoic acid increased by 82.1% (± 30.7% SD) (Figure II-6c). Under Si-limitation, higher amounts of the three PUFAs were induced, especially eicosapentaenoic acid which increased by one order of magnitude with respect to high dilution rate (from 1.41 fmol $cell^{-1}$ ± 0.11 fmol $cell^{-1}$ SD to 18.97 fmol $cell^{-1}$ ± 11.39 fmol $cell^{-1}$ SD), representing 21.0% (± 8.4% SD) of the total amount of PUA precursors (Figure II-6c).

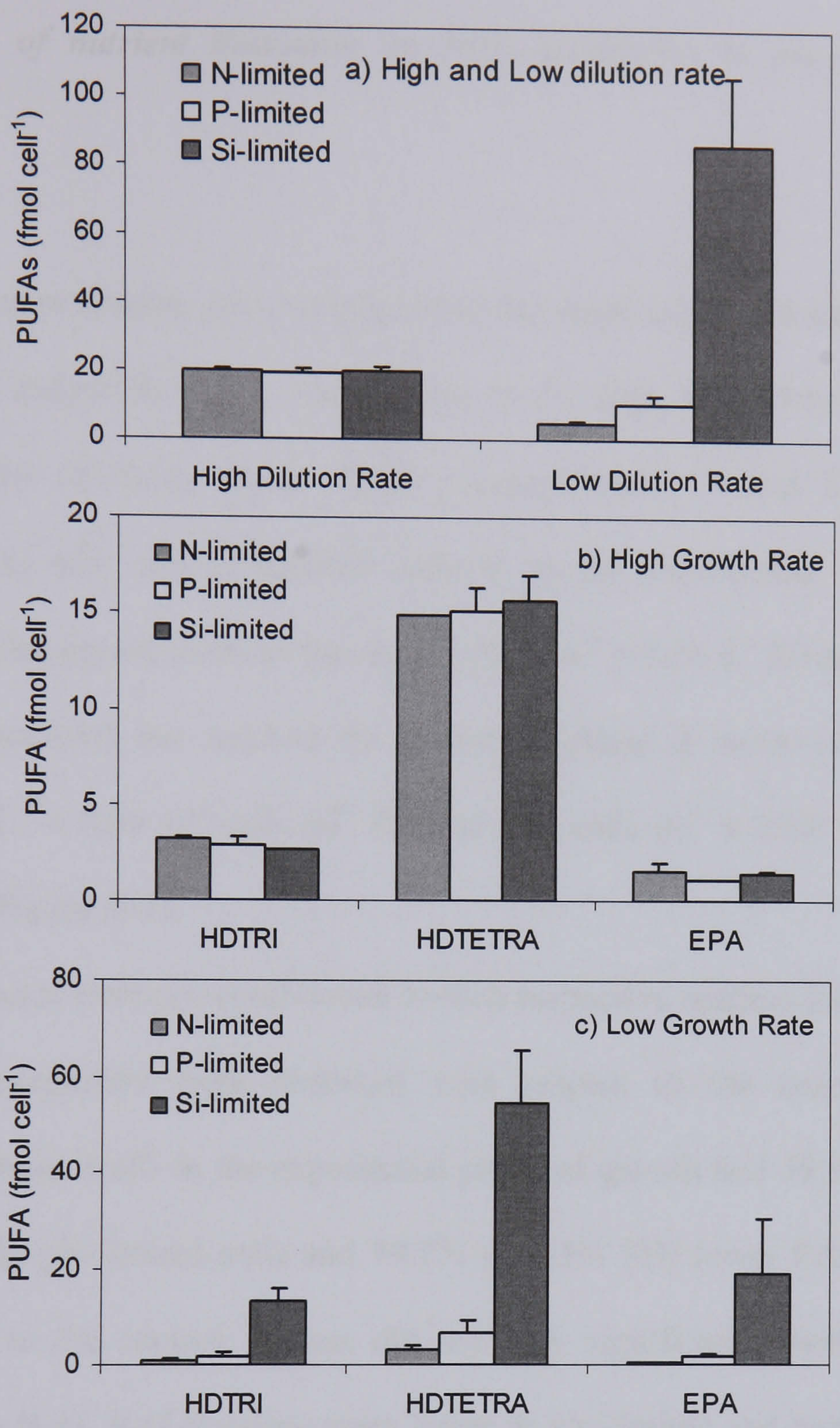


Figure II-6. PUA-precursor PUFA content per cell at at high and low dilution rate in N-limited, P-limited, and Si-limited continuous cultures of *S. marinoi.* (b) PUFA relative composition at high dilution rate, and (c) low dilution rate. Different PUFAs are indicated in the legend. EPA is eicosapentaenoic acid, HDTRI is hexadecatrienoic acid, and HDTETRA is hexadecatetraenoic acid. For all plots, data are means of replicates from 2 independent experiments. Error bars are standard deviations.

II.3.3 Effect of nutrient limitation on PUA production in pre-acclimated batch cultures

For these experiments, only samples from the exponential and stationary phases of growth were compared to the control due to the high variability in conditions of cultures in the declining phase. Both pre-acclimated N- and P-limited cultures (abbreviated as pN- and pP-limited culture) in the exponential phase of growth exhibited similar growth rates to the control (0.91 d^{-1} ± 0.04 d^{-1} SD and 0.88 d^{-1} ± 0.06 d^{-1} SD, respectively) but reached the stationary phase at lower cell concentrations (0.29 cells mL^{-1} ± 0.04 10^6 cells ml^{-1} SD and 0.22 cells ml^{-1} ± 0.02 10^6 cells ml^{-1} SD, respectively, Figure II-7).

Since cultures were pre-conditioned to their respective nutrient level, differences in cell optical properties were observed with respect to the control even in the exponential phase. Cells in the exponential phase of growth had 39.9% (± 14.4% SD) lower FALS in pN-limited cells and 59.7% (± 4.5% SD) lower FALS in pP-limited cells relative to the control. Values did not vary significantly with the age of the culture (Table II-4). RALS values were lower in pN-limited and higher in pP-limited cells in the exponential phase, while no significant difference with the control was observed in the stationary phase. Red fluorescence values were 47.4% (± 18.7% SD) lower in exponential pN-limited cells and no difference was observed in pP-limited cells as compared to the control, while in the stationary phase, pre-acclimated nutrient-limited cells showed lower values (51.8 ± 7.0% SD and 30.0 ± 7.3% SD in N- and P-limited cells respectively) (Table II-4).

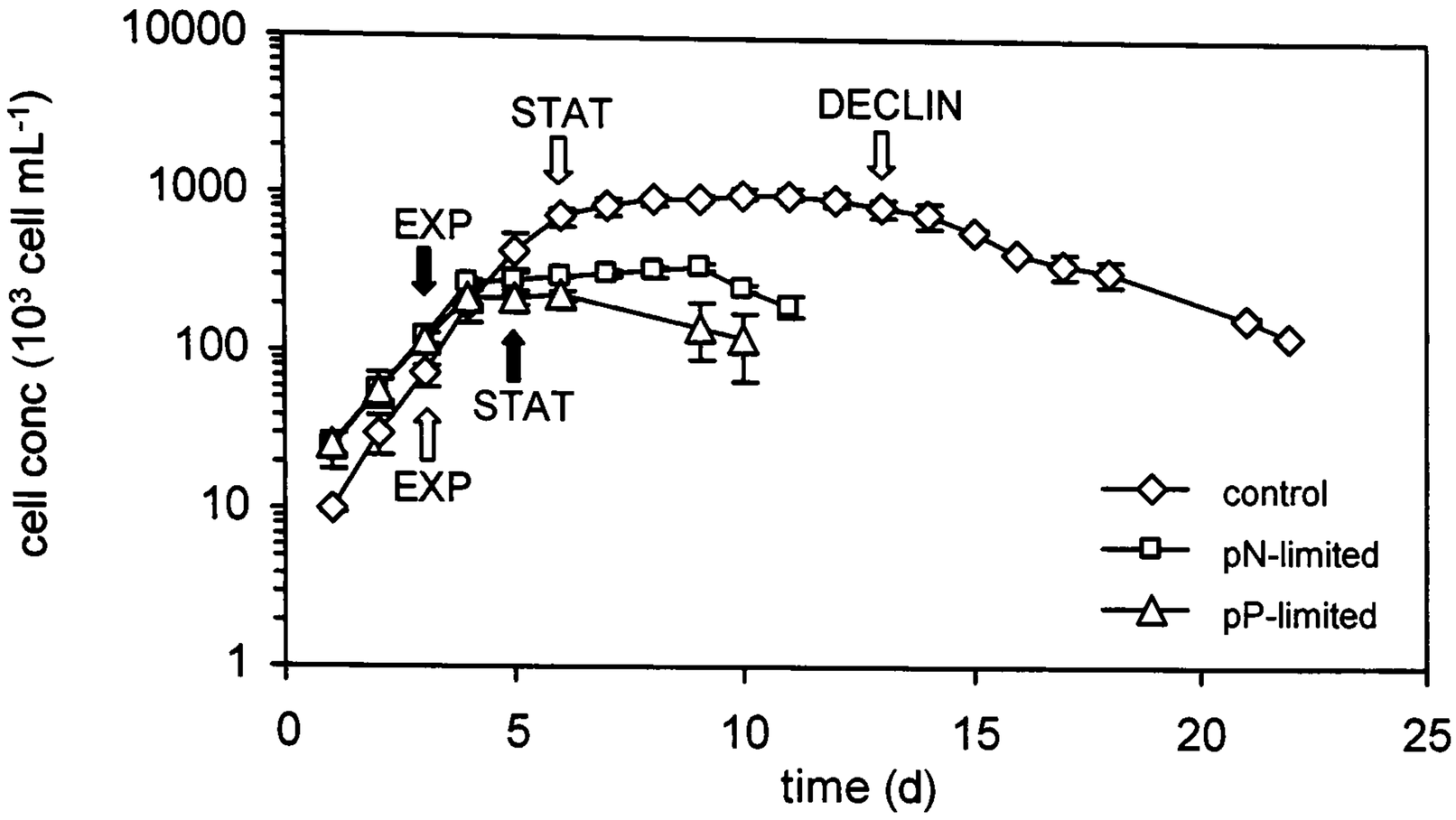


Figure II-7. Average cell concentrations of *S. marinoi* during growth in batch cultures under nutrient-replete condition (open diamonds) or in pre-acclimated nutrient-limited cultures grown under nitrogen-limited (pN-limited, open squares) and phosphate-limited (pP-limited, open triangles) growth medium. Arrows indicate sampling points for analyses of polyunsaturated aldehydes (PUAs) and their precursor polyunsaturated fatty acids (PUFAs). White arrows for the nutrient-replete and blacks arrows for nutrient-limited cultures. Error bars are standard deviations (when not visible, error bars are smaller than the symbol of the average cell concentration).

Table II-4. Values of Forward Angle Light Scatter (FALS), Right Angle Light Scatter (RALS), red fluorescence from chlorophyll (RED), all expressed in units relative to the beads used as internal standard (r. u.), chlorophyll *a* (Chl *a*), and percentage of SYTOX Green positive cells (SYTOX+, non-viable) of *S. marinoi* batch cultures in exponential (EXP) and stationary (STAT) phase under standard conditions (replete) and in pre-acclimated nutrient-limited culture grown in N-limited (pN-limited) or in phosphate-limited (pP-limited) growth medium. Data are means ± standard deviations (for the nutrient-replete conditions, $n = 9$ for cell densities, FALS, RALS, RED, and SYTOX+; $n = 4$ for Chl *a*; for the nutrient-limited conditions, $n = 3$).

Growth phase	Initial nutrient conditions	FALS (r. u.)	RALS (r. u.)	RED (r. u.)	Chl *a* (pg cell^{-1})	SYTOX+ (%)
EXP	replete	10.53 ± 1.74	0.83 ± 0.15	79.04 ± 5.16	0.76 ± 0.17	0.7 ± 0.1
EXP	pN-limited	6.33 ± 1.51	0.50 ± 0.04	41.61 ± 13.76	0.16 ± 0.02	1.4 ± 0.4
EXP	pP-limited	4.25 ± 0.48	1.14 ± 0.12	78.81 ± 8.37	0.24 ± 0.09	0.8 ± 0.3
STAT	replete	9.50 ± 1.38	1.09 ± 0.30	116.26 ± 12.67	1.01 ± 0.14	3.6 ± 0.8
STAT	pN-limited	5.76 ± 0.51	1.03 ± 0.06	56.05 ± 9.18	0.25 ± 0.03	9.2 ± 0.8
STAT	pP-limited	5.19 ± 0.47	1.07 ± 0.03	81.36 ± 10.51	0.22 ± 0.02	15.9 ± 3.1

Cell viability was not affected by nutrient-limited conditions in the exponential phase, while a higher proportion of SYTOX Green-positive cells was observed in the stationary phase as compared to the control (Table II-4).

POC, PON, and Particulate Organic Phosphorus (POP) per cell and the N:P ratio were strongly affected by previous N- and P-limitation (Table II-5) as also Chl *a* content, which was reduced by 79% and 69% in the exponential phase, respectively (Table II-5). Reduction in Chl *a* content and the ratio ratio between organic nutrient content are typical responses of nutrient-limited cells which undergo metabolic modifications under nutrient stress (for review, Wilhelm et al. 2006).

Total PUAs produced per cell in exponentially growing cultures were slightly higher in pP-limited cultures (1.61 fmol $cell^{-1}$ ± 0.59 fmol $cell^{-1}$ SD), as compared to the control (1.23 fmol $cell^{-1}$ ± 0.37 fmol $cell^{-1}$ SD) (Figure II-8a), mainly due to an increase in heptadienal (Figure II-8b), but these differences were not significant (Student *t*-test, $p>0.05$). In pN-limited cultures, PUAs produced per cell were significantly lower (Student *t*-test, $p<0.001$) in exponential phase due to a decrease of both heptadienal and octadienal (Figure II-8a and b).

Table II-5. Particulate organic nitrogen (PON), phosphate (POP), or carbon (POC), molar ratios of N:P, and C:N of *S. marinoi* batch cultures in exponential (EXP) and stationary (STAT) phase under standard conditions (replete) and in pre-acclimated nutrient-limited cultures grown in N-limited (pN-limited) or in phosphate-limited (pP-limited) growth medium. Data are means ± standard deviations (n = 9 for the nutrient-replete conditions, n = 3 for the nutrient-limited conditions).

		replete	pN-limited	pP-limited
EXP	PON (pg cell^{-1})	2.35 ± 0.16	0.87 ± 0.09	1.55 ± 0.21
	POP (pg cell^{-1})	0.39 ± 0.04	0.25 ± 0.05	0.12 ± 0.02
	POC (pg cell^{-1})	12.85 ± 1.35	6.15 ± 0.19	8.42 ± 1.34
	N:P	13.40 ± 1.12	7.71 ± 0.98	28.44 ± 1.73
	C:N	6.40 ± 0.76	8.28 ± 1.02	6.31 ± 0.33
STAT	PON (pg cell^{-1})	2.29 ± 0.23	0.96 ± 0.08	1.69 ± 0.20
	POP (pg cell^{-1})	0.34 ± 0.04	0.32 ± 0.03	0.08 ± 0.01
	POC (pg cell^{-1})	15.71 ± 2.17	10.63 ± 1.06	15.61 ± 1.42
	N:P	16.68 ± 1.05	6.75 ± 1.10	45.82 ± 2.50
	C:N	8.02 ± 1.13	12.91 ± 0.68	10.83 ± 0.41

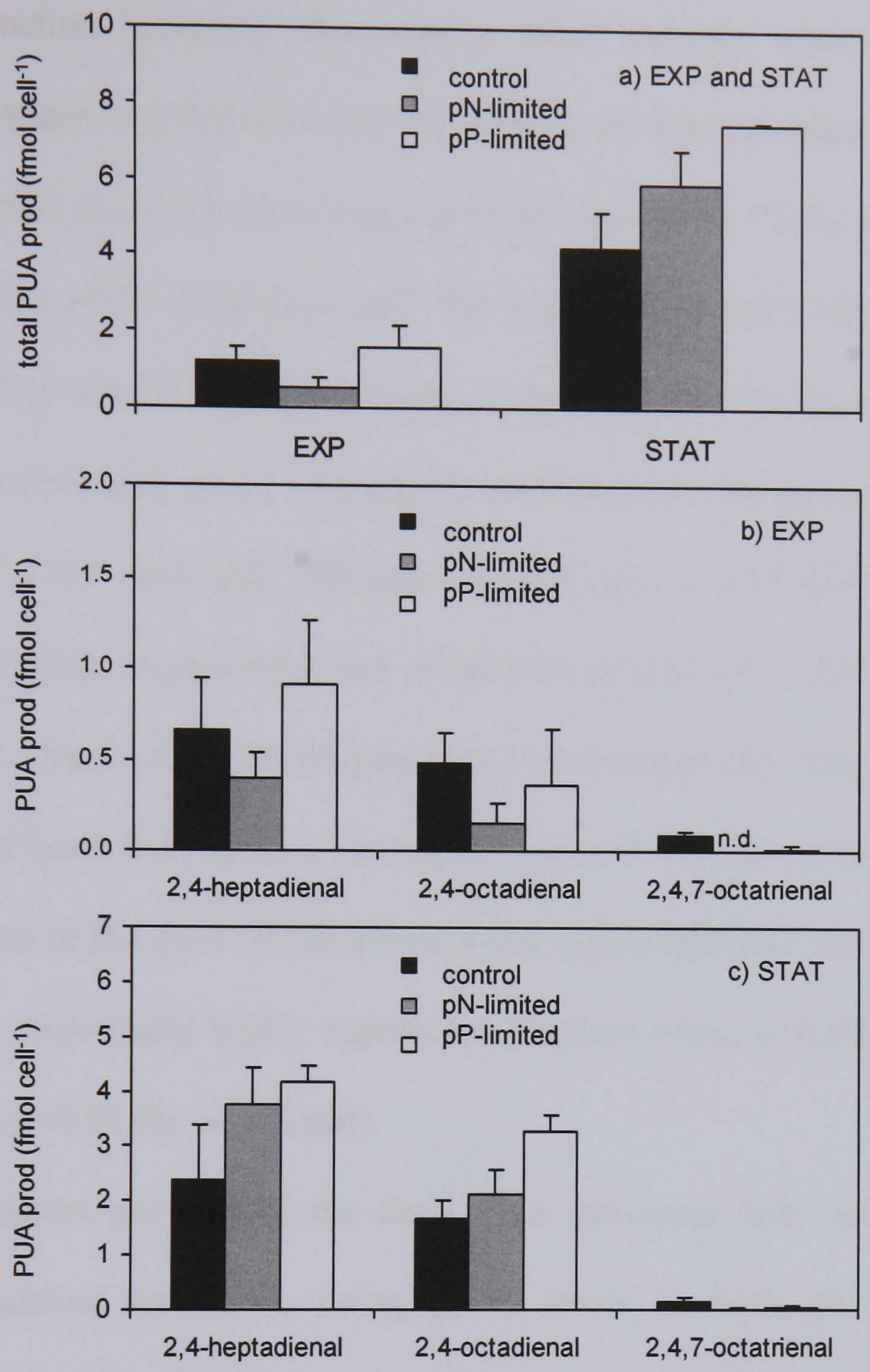


Figure II-8. (a) Total PUA production per cell in *S. marinoi* batch cultures in exponential (EXP) and stationary (STAT) phases of growth in nutrient-replete condition (control), or in pre-acclimated nutrient-limited cultures grown under nitrogen-limited (pN-limited) and phosphate-limited (pP-limited) growth medium (b) Composition in terms of different PUAs produced at the three culture conditions in exponential phase of growth. (c) Composition in terms of different PUAs produced at the three culture conditions in stationary phase of growth. Error bars are standard deviations. n.d. means not detected.

PUA production increased dramatically when cultures attained the stationary phase, by 10.5 times in pN-limited and 4.6 times in pP-limited cultures, in comparison to the exponential phase (Student *t*-test, $p<0.001$ for both). PUAs produced were as high as 7.49 fmol $cell^{-1}$ ± 0.08 fmol $cell^{-1}$ SD in pP-limited and 5.90 fmol $cell^{-1}$ ± 0.94 fmol $cell^{-1}$ SD in the pN-limited cultures (Figure II-8c). The increase in PUAs in cultures in the stationary phase was mainly attributable to an increase in heptadienal (3.8 fmol $cell^{-1}$ ± 0.7 fmol $cell^{-1}$ SD and 4.2 fmol $cell^{-1}$ ± 0.15 fmol $cell^{-1}$ SD in pN- and pP-limited cells, respectively) and octadienal production in pP-limited cells (3.3 fmol $cell^{-1}$ ± 0.1 fmol $cell^{-1}$ SD) (Figure II-8c). Octatrienal was only present in traces in all cultures (Figure II-8b and c). For both pN-and pP-limited cultures, the difference in the production of the three PUAs between the exponential and the stationary phases of growth was statistically highly significant (Student *t*-test, $p<0.001$ for heptadienal and octadienal, $p<0.01$ for octatrienal).

Average content per cell of the three PUA precursor fatty acids was strongly affected by nutrient limitation, being much lower in both pN- and pP-limited exponential and stationary cultures as compared to the control (Figure II-9a). No increase of these PUFAs occurred between exponential and stationary growth phases in cultures pre-acclimated to nutrient limitation (Student *t*-test, $p>0.1$). However, pN- and pP-limited cultures in the stationary phase contained 6.9 fmol $cell^{-1}$ ± 4.2 fmol $cell^{-1}$ SD and 7.7 fmol $cell^{-1}$ ± 1.7 fmol $cell^{-1}$ SD of the three PUFAs, respectively, as compared to 24.7 fmol $cell^{-1}$ ± 8.5 fmol $cell^{-1}$ SD of the control cultures (Figure II-9a).

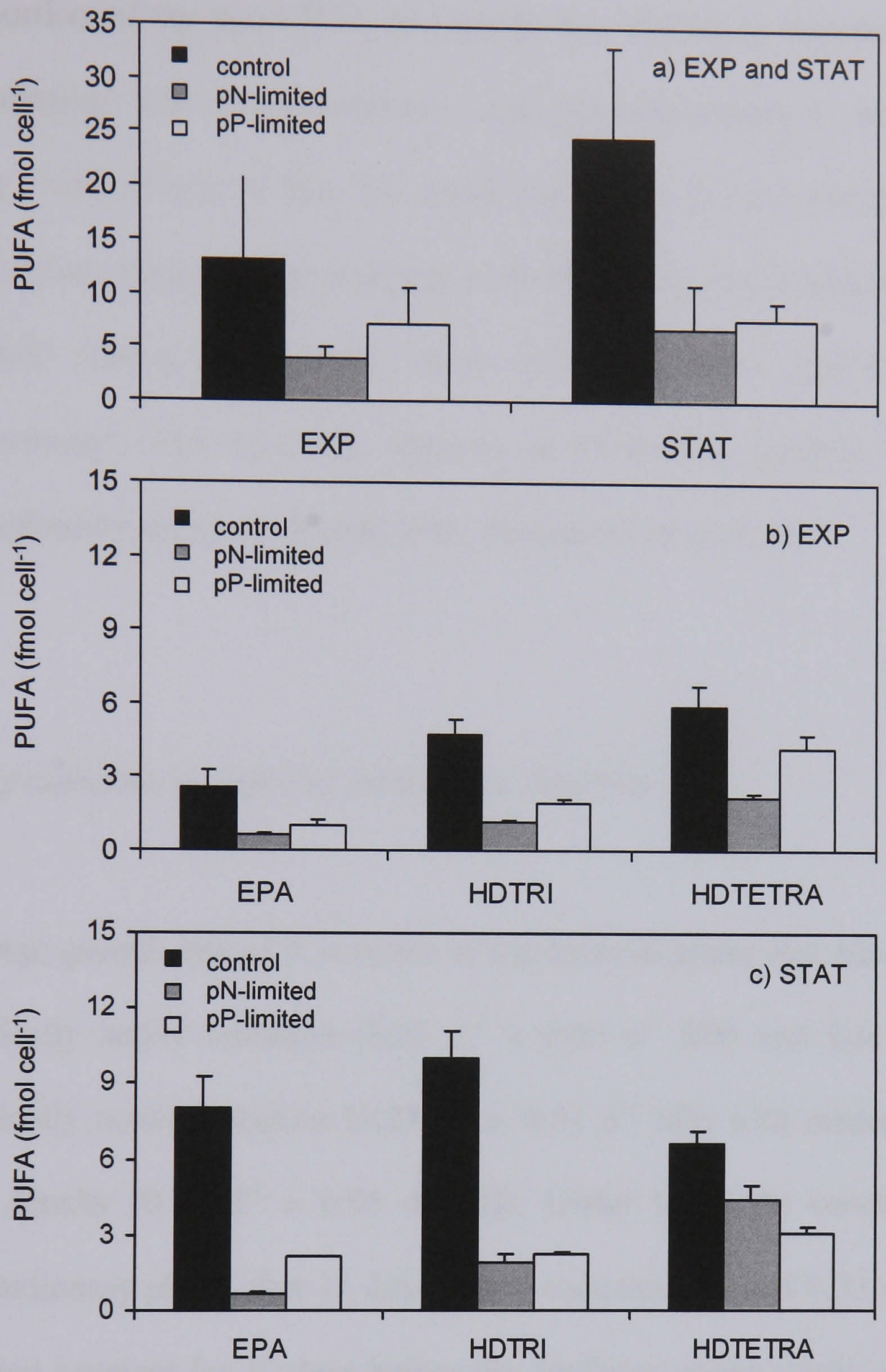


Figure II-9. (a) PUA-precursor PUFA cell content in S. marinoi batch cultures in exponential (EXP) or stationary (STAT) phases of growth under nutrient-replete (control), or in pre-acclimated nutrient-limited cultures grown under nitrogen-limited (pN-limited) and phosphate-limited (pP-limited) growth medium. (b) PUFA relative composition in the exponential, and (c) in the stationary phases of growth. Different PUFAs are indicated in the legend. EPA is eicosapentaenoic acid, HDTRI is hexadecatrienoic acid, and HDTETRA is hexadecatetraenoic acid. For all plots, data are means of replicates from 9 independent experiments for the control and from 3 independent experiments for the nutrient-limited cultures. Error bars are standard deviations.

The proportion of the three PUA precursors was similar in exponential pN- and pP-limited cultures, with hexadecatrienoic and hexadecatetraenoic acids being the dominant fatty acids (Figure II-9b). The trend was similar to the controls in stationary pP-limited cultures, with eicosapentaenoic acid increasing and hexadecatrienoic acid decreasing their relative contributions (from 13.7% to 28.8%, and from 58.8% to 43.6%, respectively). The trend was opposite in pN-limited cultures, but variations were not significantly different (Figure II-9c, Student *t*-test, $p>0.1$).

II.3.4 PUA production at different photon flux densities

The average growth rate of *S. marinoi* in exponential phase was slower under low photosynthetically active radiation (0.23 d^{-1} ± 0.05 d^{-1} SD) and faster under high photosynthetically active radiation (1.27 d^{-1} ± 0.01 d^{-1} SD) with respect to standard photon flux density (0.92 d^{-1} ± 0.05 d^{-1} SD). Under low light condition, cultures reached the stationary phase after 11 days at cell concentrations of 0.33 x 10^6 cell mL^{-1}, and remained constant for 10 days before the declining phase, while under standard growth condition, cultures reached stationary phase at day 5 with cell concentrations of 1.16 x 10^6 cell mL^{-1} (Figure II-10). Cell concentrations under high light condition reached maximum cell concentrations of 1.06 x 10^6 cell mL^{-1} at day 4 and then decreased afterwards (Figure II-10).

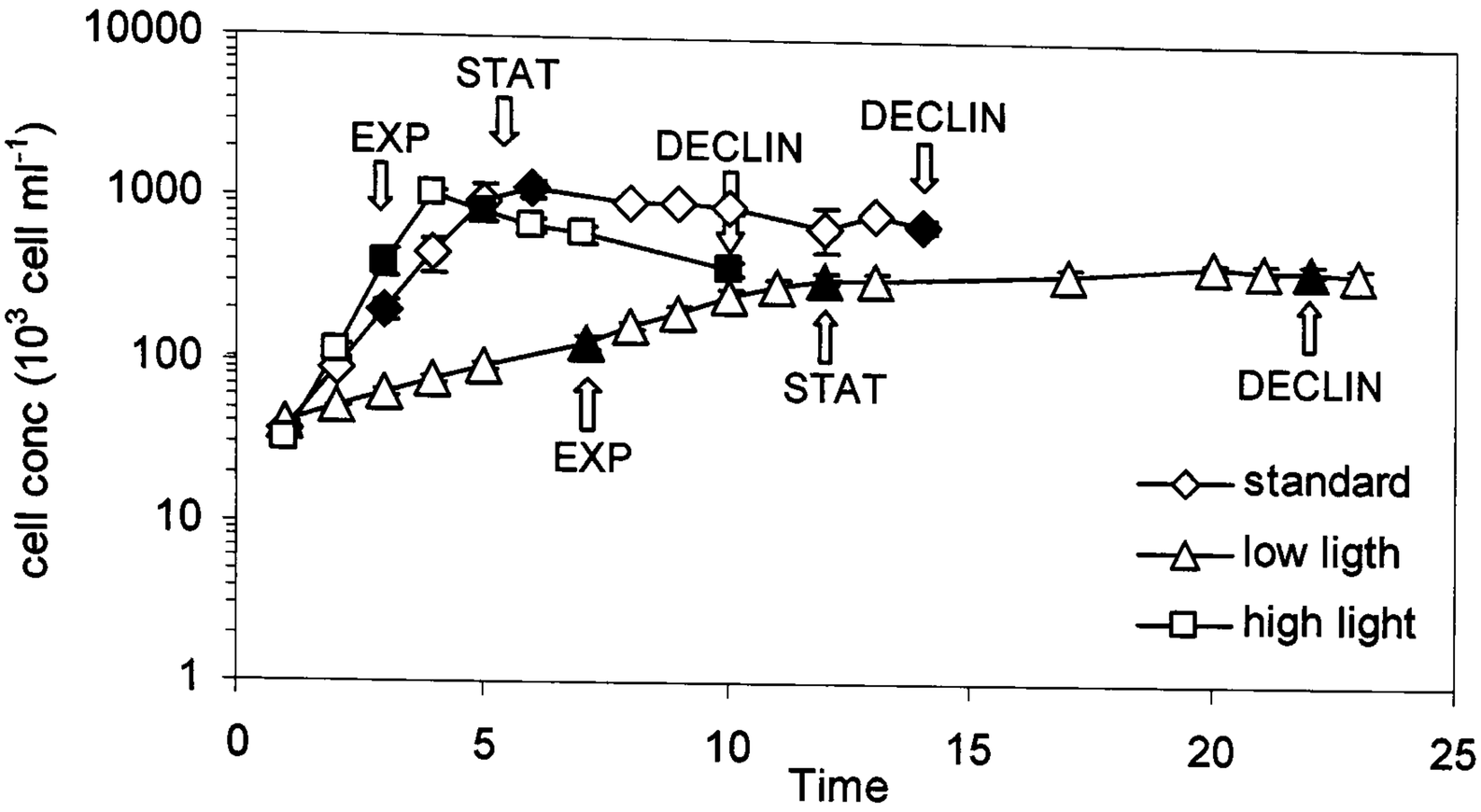


Figure II-10. Average cell concentrations of *S. marinoi* under photosynthetically active radiation of 150 µmol quanta m^{-2} s^{-1}(standard used as control, open diamonds), 10 µmol quanta m^{-2} s^{-1} (low light, open triangles) and 450 µmol quanta m^{-2} s^{-1} (high light, open squares). Arrows indicate sampling points (in black) for analyses of polyunsaturated aldehydes and their precursor polyunsaturated fatty acids. EXP, STAT, and DECLIN indicate the exponential, stationary, and declining phase of growth, respectively. Data are means of replicates from 4 independent experiments for the control, while for low and high photon flux densities, n = 3. Error bars are standard deviations (when not visible, error bars are smaller than the symbol of the average cell concentration).

In low-light acclimated cells, FALS, RALS and Particulate Organic Carbon (POC) were lower while Chl *a* was higher than the control at the three phases of growth (Tukey Multiple Comparison post-test, $p<0.05$) (Table II-6) while no difference was observed for the red fluorescence (ANOVA, $p>0.05$). In high-light acclimated cells, red fluorescence and Chl *a* were lower and POC and RALS were higher as compared to the control at the three phases of growth (Tukey Multiple Comparison post-test, $p<0.001$), while FALS was similar to the control (ANOVA, $p>0.5$) (Table II-6). Increasing Chl *a* content and decreasing POC and cell volume with decreasing photosynthetically active radiation is a typical response of diatoms to photoacclimation (Anning et al. 2000, Sarthou et al. 2005). The high red fluorescence with respect to Chl *a* content observed in low-light acclimated cells may be due to the reduction in cell volume (estimated by FALS) that increased the red fluorescence per cell (Sosik et al. 1989).

In all culture conditions, RALS and POC increased from mid-exponential to early declining cell phase, red fluorescence and Chl *a* increased from exponential to stationary phase and then decreased in declining phase (Table II-6). FALS remained stable during the whole growth cycle under low light condition while it decreased under standard and high light condition in declining cell phase of growth (Table II-6). The proportion of SYTOX+ cells was very low in the exponential phase while it increased with culture age and reached 32.9% (±7.3% SD), 15.7% (± 4.1% SD) and 10.8% (± 1.6% SD) in cultures acclimated to low, standard and high photon flux densities, respectively (Table II-6).

Table II-6. Values of Forward Angle Light Scatter (FALS), Right Angle Light Scatter (RALS), red fluorescence from chlorophyll (RED), all expressed in units relative to the beads used as internal standard (r. u.), chlorophyll *a* (Chl *a*), particulate organic carbon (POC), and percentage of SYTOX Green positive cells (SYTOX+, non-viable) during different phases of *S. marinoi* cultures grown under low (10 µmol quanta m^{-2} s^{-1}), standard (150 µmol quanta m^{-2} s^{-1}, used as a control) and high photosynthetically active radiation (450 µmol quanta m^{-2} s^{-1}). EXP is exponential, STAT is stationary, DECLIN is declining. Data are means ± standard deviations (n = 4 for the control and n =3 for low and high photon flux densities).

Growth phase	Light conditions	FALS (r. u.)	RALS (r. u.)	RED (r. u.)	Chl *a* (pg $cell^{-1}$)	POC (pg $cell^{-1}$)	SYTOX+ (%)
EXP	low light	3.60 ± 0.07	0.37 ± 0.01	87.63 ± 3.03	1.68 ± 0.46	6.02 ± 1.50	2.7 ± 0.5
EXP	standard	6.24 ± 0.71	0.51 ± 0.09	87.29 ± 11.02	1.16 ± 0.12	12.40 ± 2.09	0.7 ± 0.1
EXP	high light	6.96 ± 0.90	0.58 ± 0.03	44.84 ± 1.38	0.10 ± 0.04	18.24 ± 1.89	1.0 ± 0.5
STAT	low light	3.71 ± 0.04	0.37 ± 0.02	102.17 ± 3.90	3.09 ± 0.16	6.11 ±0.91	5.6 ± 0.8
STAT	standard	6.04 ± 0.34	0.69 ± 0.33	121.20 ± 9.31	1.50 ± 0.25	14.02 ± 4.47	3.0 ± 1.5
STAT	high light	6.47 ± 0.50	0.75 ± 0.05	38.68 ± 1.11	0.13 ± 0.01	24.33 ± 3.88	1.5 ± .2
DECLIN	low light	3.95 ± 0.49	0.57 ± 0.04	99.49 ± 22.49	3.22 ± 0.07	9.41 ± 0.94	32.9 ± 7.3
DECLIN	standard	4.93 ± 0.92	0.94 ± 0.44	59.91 ± 7.32	1.24 ± 0.24	14.45 ±1.82	15.7 ± 4.1
DECLIN	high light	5.52 ± 0.16	1.56 ± 0.11	27.52 ± 4.43	0.08 ± 0.02	26.53 ± 4.13	10.8 ± 1.6

No significant change was observed for PUA production between high and standard light condition at the different growth phases (ANOVA, $p>0.1$) while a significant decrease was observed under low light condition in stationary and declining phase of growth with respect to the two other light conditions (Tukey Multiple Comparison post-test, $p<0.05$) (Figure II-11). The amount of PUA produced by cells in the exponential phase of growth were similar among the three growth conditions (1.63 fmol $cell^{-1}$ ± 0.32 fmol $cell^{-1}$ SD, 1.97 fmol $cell^{-1}$ ± 0.41 fmol $cell^{-1}$ SD, 2.01 fmol $cell^{-1}$ ± 0.24 fmol $cell^{-1}$ SD, for control, high and low light respectively) (ANOVA, $p>0.5$) (Figure II-11a). In the stationary growth phase, PUA production was lower in low light acclimated cells (1.64 fmol $cell^{-1}$ ± 0.09 fmol $cell^{-1}$ SD) as compared to the control, mainly due to a decrease of both 2,4-heptadienal and 2,4-octadienal (Figure II-11b), while it increased under standard and high light conditions (5.00 fmol $cell^{-1}$ ± 0.50 fmol $cell^{-1}$ SD and 5.30 fmol $cell^{-1}$ ± 0.38 fmol $cell^{-1}$ SD, respectively).

PUA production remained stable between early stationary and declining phase of growth for the three photon flux densities (Student *t*-test, $p< 0.05$). Since Chl *a* per cell was strongly affected by light conditions, PUA production normalized by Chl *a* content was higher and lower in high and low light acclimated cells, respectively, as compared to the control (Tukey Multiple Comparison post-test, $p< 0.05$). The opposite trend was observed when normalized by POC per cell (Tukey Multiple Comparison post-test, $p< 0.001$) since POC increased with decreasing Chl *a* cell content (Table II-6)

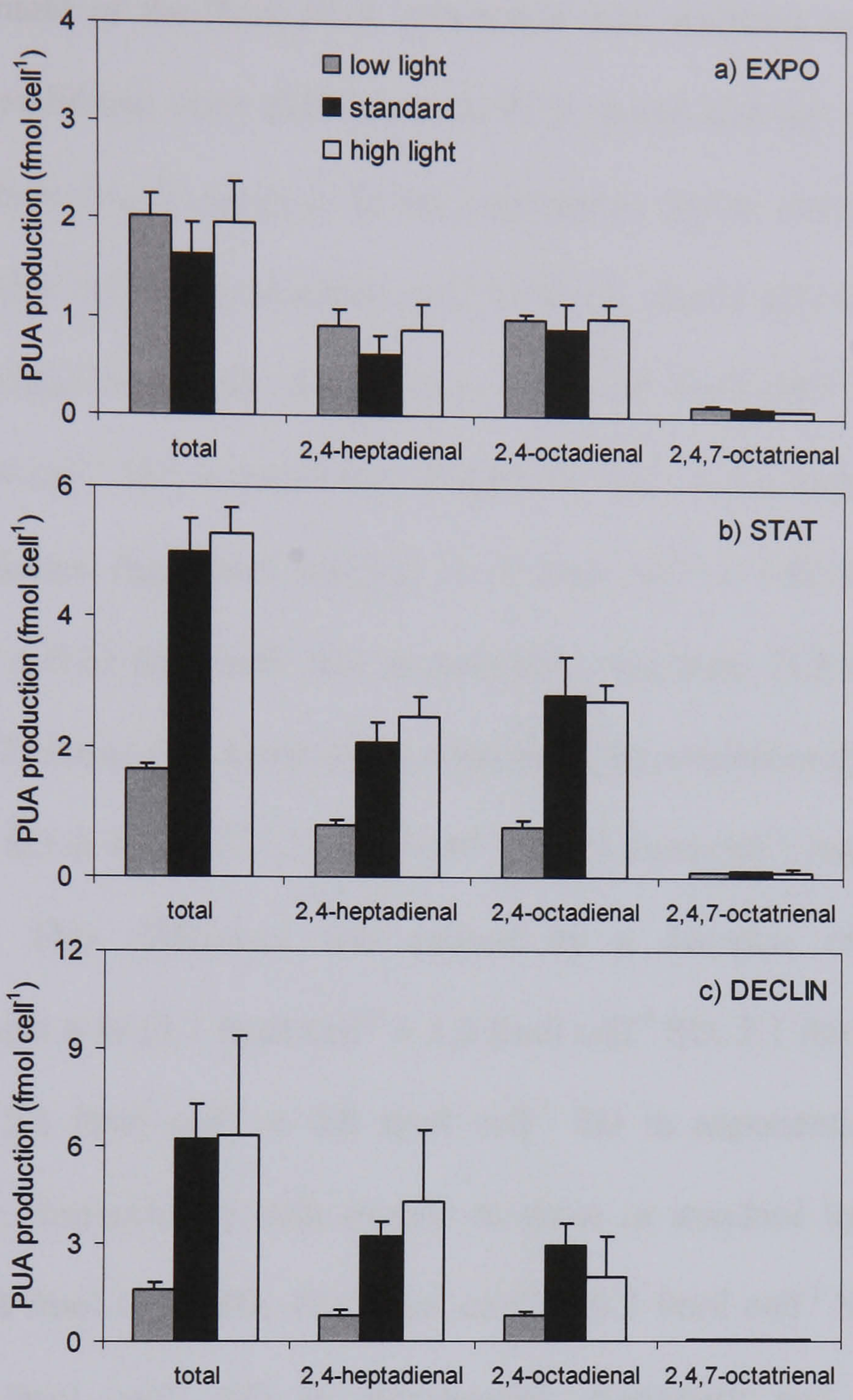


Figure II-11. Total PUA production per cell and composition in term of PUAs (2,4-heptadienal, 2,4-octadienal and 2,4,7-octatrienal) of *S. marinoi* (a) in exponential (EXPO), (b) stationary (STAT) and (c) declining (DECLIN) phases of growth in *S. marinoi* under an irradianceof 10 µmol quanta m^{-2} s^{-1} (low light), 150 µmol quanta m^{-2} s^{-1}(standard), and 450 µmol quanta m^{-2} s^{-1} (high light). Error bars are standard deviations (when not visible, error bars are smaller than the symbol of the average cell concentration). N = 4 for the control, n = 3 for low and high photon flux densities.

The cell content of the three PUA precursors was analyzed only under low and standard light conditions, since differences in PUA production per cell were observed only between these two conditions. In the exponential phase, the cell content of the three PUFAs under low light condition was 56.6% (± 20.0% SD) lower as compared to the standard light conditions (4.82 fmol $cell^{-1}$ ± 2.22 fmol $cell^{-1}$ SD and 11.1 fmol $cell^{-1}$ ± 1.7 fmol $cell^{-1}$ SD, respectively) (Figure II-12a). In the early stationary phase and declining phases, their level reached 10.17 fmol $cell^{-1}$ ± 1.92 fmol $cell^{-1}$ SD and 7.89 fmol $cell^{-1}$ ± 0.23 fmol $cell^{-1}$ SD, respectively, and were 33.8% (12.4% SD) and 69.1% (0.1% SD) lower than those under standard light conditions (15.37 fmol $cell^{-1}$ ± 0.65 fmol $cell^{-1}$ SD in the and 25.51 fmol $cell^{-1}$ ± 5.53 fmol $cell^{-1}$, respectively) (Figure II-12b and c). This difference was caused by a decrease of the amount of hexadecatetraenoic acid (2.7 fmol $cell^{-1}$ ± 1.5 fmol $cell^{-1}$ SD, 5.1 fmol $cell^{-1}$ ± 0.8 fmol $cell^{-1}$ SD, and 5.2 fmol $cell^{-1}$ ± 0.8 fmol $cell^{-1}$ SD in exponential, stationary and declining phase, respectively) with respect to those in standard light condition (7.8 fmol $cell^{-1}$ ± 0.8 fmol $cell^{-1}$ SD, 12.1 fmol $cell^{-1}$ ± 0.3 fmol $cell^{-1}$ SD, and 20.0 fmol $cell^{-1}$ ± 3.72 fmol $cell^{-1}$ SD in exponential, stationary and declining phase, respectively) (Figure II-12b and c).

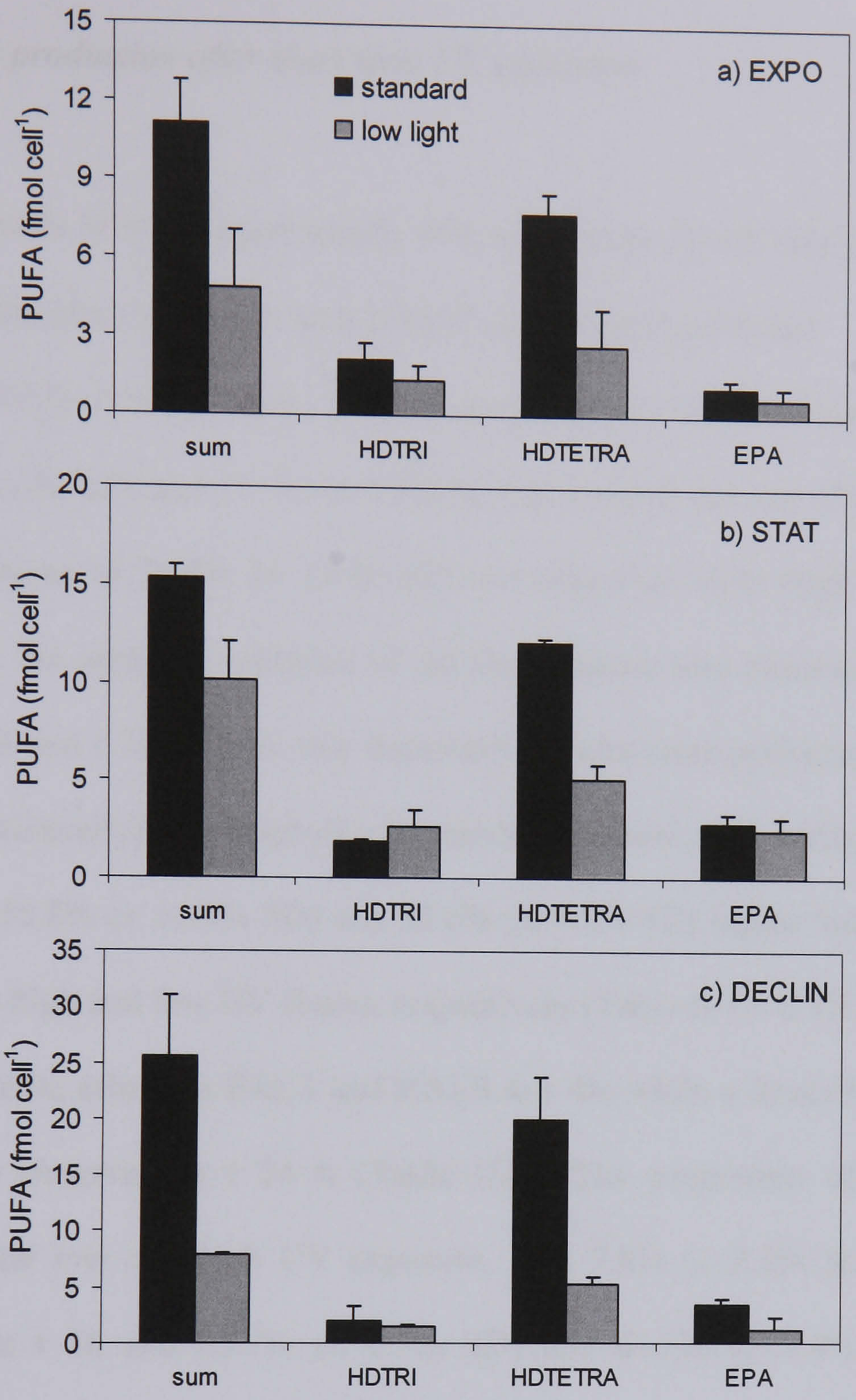


Figure II-12. (a) PUA-precursor PUFAs in exponential (EXP) or stationary (STAT) and declining (DECLIN) phases of growth in *S. marinoi* under an irradianceof 150 µmol quanta m^{-2} s^{-1}(standard) and 10 µmol quanta m^{-2} s^{-1} (low light); (b) PUFA relative composition in the exponential; (c) in stationary; and (d) in the declining phase of growth. Different PUFAs are indicated in the legend. EPA is eicosapentaenoic acid, HDTRI is hexadecatrienoic acid, and HDTETRA is hexadecatetraenoic acid. Error bars are standard deviations. N = 2 for the control, and n = 3 for low photon flux density.

II.3.5 PUA production after short term UV exposures

Control cells divided exponentially with an average growth rate of 1.01 d^{-1} (± 0.01 d^{-1} SD), while cells exposed to both UVB fluxes stopped growing (

Figure *II-13*). After 4 h from UV exposure, the cell red fluorescence increased by 35.8% (± 11.3% SD) and 17.5% (± 7.5% SD) in control and low UVB exposed cells, while a decrease of 22.8% (± 2.1% SD) was observed under high UVB conditions (Table II-7). No apparent variation of red fluorescence was observed in treated cells between t 4h and t 24h (since only duplicate cultures were performed, data were not analyzed statistically). Chlorophyll *a* increased in control cells during the experiment, resulting in 55.8% (± 10.9% SD) and 32.6% (± 9.5% SD) higher values after 24 h as compared to high and low UV fluxes, respectively (Table II-7). UVB exposure did not have any visible effect on FALS and RALS at t 4h, while a lower FALS and higher RALS were observed at t 24 h (Table II-7). The proportion of SYTOX+ cells increased after low and high UV exposure, with 7.8% (± 2.3% SD) and 15.9% (± 5.2% SD) at t 4h and 25.7% (± 1.7% SD) and 61.3% (± 7.9% SD) after 24h, respectively for the two UV fluxes (Table II-7).

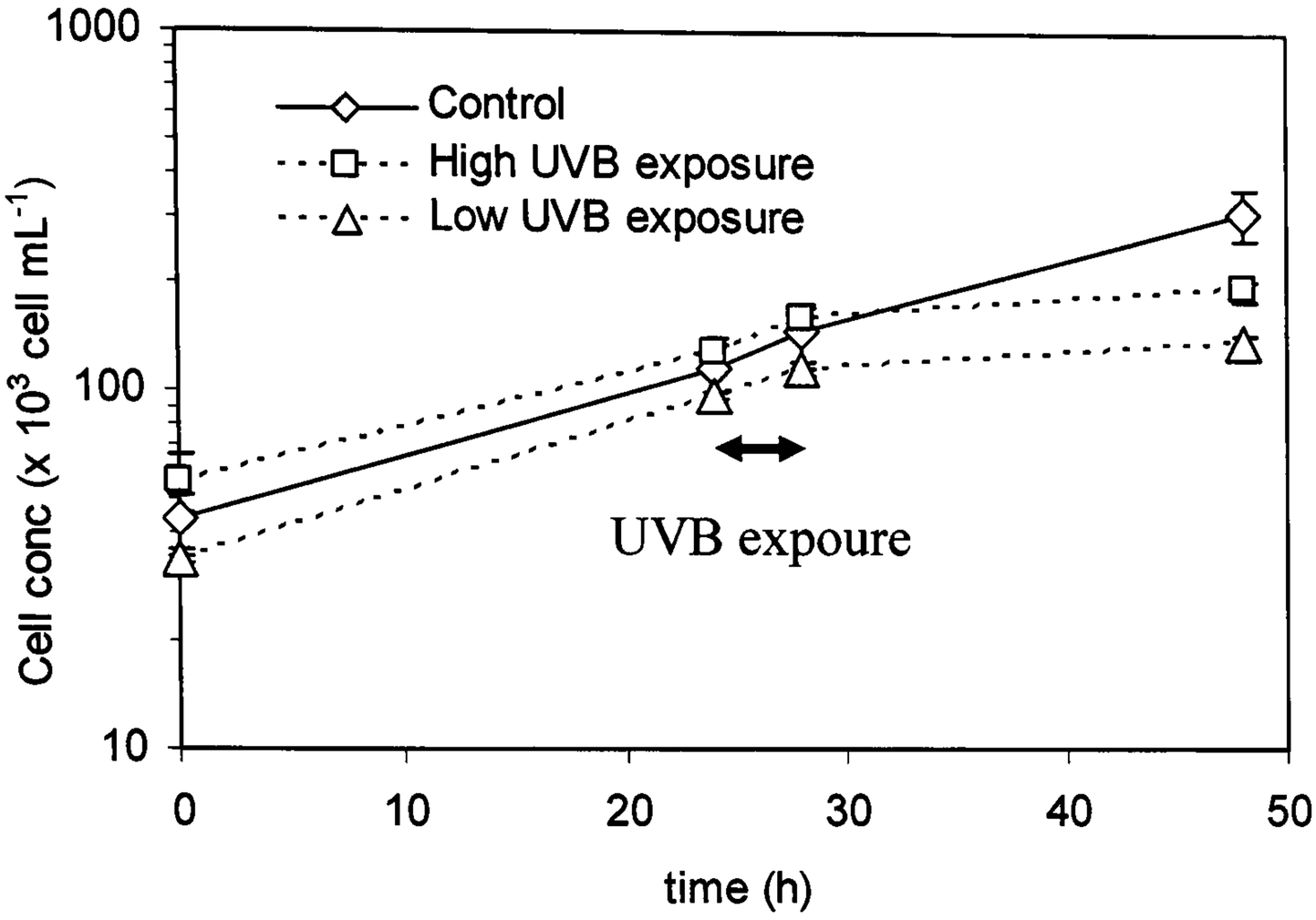


Figure II-13. Average cell concentrations of *S. marinoi* under standard growth conditions (control) or exposed to 4 h UVB at 0.15 W m^{-2} s^{-1} (High) 0.05 W m^{-2} s^{-1} (Low). Samples were taken before UV exposure, just after UV exposure and 24 h later. Error bars are standard deviation (when not visible, error bars are smaller than the symbol of the average cell concentration). N = 2.

Table II-7. Values of Forward Angle Light Scatter (FALS), Right Angle Light Scatter (RALS), red fluorescence from chlorophyll (RED), all expressed in units relative to the beads used as internal standard (r. u.), chlorophyll *a* content (Chl *a*), and percentage of SYTOX Green positive cells (SYTOX+, non-viable) of *S. marinoi* grown under standard growth conditions or exposed for 4 h to low and high UVB exposures (0.05 W m^{-2} $s^{-1}$and 0.15 W m^{-2} s^{-1}, respectively) at t 0h, 4h and 24h. Data are means ± standard deviations ($n = 2$).

Time (h)	Growth conditions	FALS (r. u.)	RALS (r. u.)	RED (r. u.)	Chl *a* (pg $cell^{-1}$)	SYTOX+ (%)
0	control	5.02 ± 0.02	0.88 ± 0.01	104.60 ± 0.50	1.10 ± 0.18	0.7 ± 0.2
	low UVB	5.13 ± 0.05	0.90 ± 0.14	95.46 ± 2.39	1.06 ± 0.34	0.9 ± 0.1
	high UVB	4.83 ± 0.10	0.84 ± 0.01	111.55 ± 1.46	1.18 ± 0.34	1.3 ± 0.5
4	control	5.56 ± 0.02	0.83 ± 0.01	141.94 ± 0.62	1.31 ± 0.14	1.0 ± 0.1
	low UVB	5.67 ± 0.09	0.89 ± 0.02	112.19 ± 5.11	1.18 ± 0.28	7.8 ± 2.3
	high UVB	5.26 ± 0.13	0.82 ± 0.02	86.08 ± 1.29	0.90 ± 0.18	15.9 ± 5.2
24	control	4.93 ± 0.02	0.73 ± 0.01	117.49 ± 0.52	1.70 ± 0.32	0.9 ± 0.1
	low UVB	4.50 ± 0.10	1.10 ± 0.01	111.28 ± 1.17	1.15 ± 0.16	25.7 ± 1.7
	high UVB	3.35 ± 0.15	1.00 ± 0.06	88.88 ± 2.12	0.75 ± 0.19	61.3 ± 7.9

Total PUA production in control cells remained constant during the period of the experiment, with an average value of 1.30 fmol $cell^{-1}$ (± 0.03 fmol $cell^{-1}$ SD) (Figure II-14). When exposed to UVB, PUA production per cell increased and reached 1.84 fmol $cell^{-1}$ (± 0.22 fmol $cell^{-1}$ SD) and 4.25 fmol $cell^{-1}$ (± 1.26 fmol $cell^{-1}$ SD) at t 4h in low and high UV exposed cells respectively (Figure II-14a). At t 24 h, PUA production reached 3.58 fmol $cell^{-1}$ (± 0.68 fmol $cell^{-1}$ SD) and 7.03 fmol $cell^{-1}$ (± 0.85 fmol $cell^{-1}$ SD) in low and high UV-exposed cultures, respectively, which represented an increase of 361.6% ± 12.5% SD and 416.2% ± 16.4% SD, respectively, as compared to t 0h (Figure II-14a). The higher PUA production after UV exposure was mainly due to an increase of both 2,4-heptadienal and 2,4-octadienal (Figure II-14b, c and d). The same trend was observed when the amount of PUAs was normalized by Chl *a* (data not shown).

Average content per cell of the three PUFAs did not follow the same pattern than that observed for PUAs. The amount of the three PUFAs remained constant in control cells, with an average value of 11.98 fmol $cell^{-1}$ (± 0.03 fmol $cell^{-1}$ SD), while it increased and reached 17.50 fmol $cell^{-1}$ (± 0.22 fmol $cell^{-1}$ SD) and 29.29 fmol $cell^{-1}$ (± 4.13 fmol $cell^{-1}$ SD) at t 4h in low and high UV exposed cells, respectively (Figure II-15a), mainly due to an increase of hexadecatetraenoic and hexadecatrienoic acids in both cases (Figure II-15b, c, and d). The total amount of the three PUFAs remained constant between t 4h and t 24h under UV exposure (Figure II-15a).

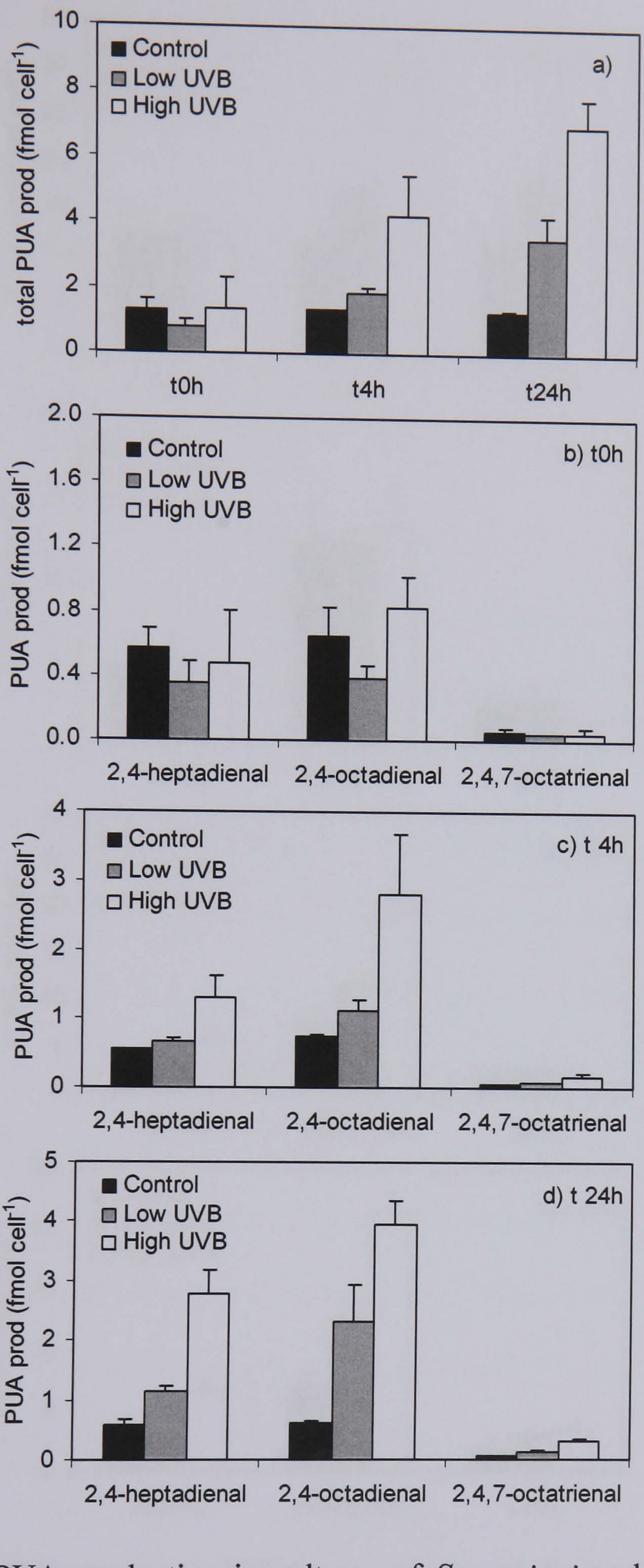


Figure II-14. (a) PUA production in cultures of *S. marinoi* under standard growth conditions (control) or exposed to 4 h-UVB at 0.05 W m^{-2} s^{-1} (Low) and 0.15 W m^{-2} s^{-1} (High). Samples were taken just before UV exposure (t 0h), just after UV exposure (t 4h) and 20 h later (t 24h). Composition in terms of different PUAs produced at the three culture conditions at t 0h (b) at t 4h (c) and at t 24h (d). Error bars are standard deviations. N = 2.

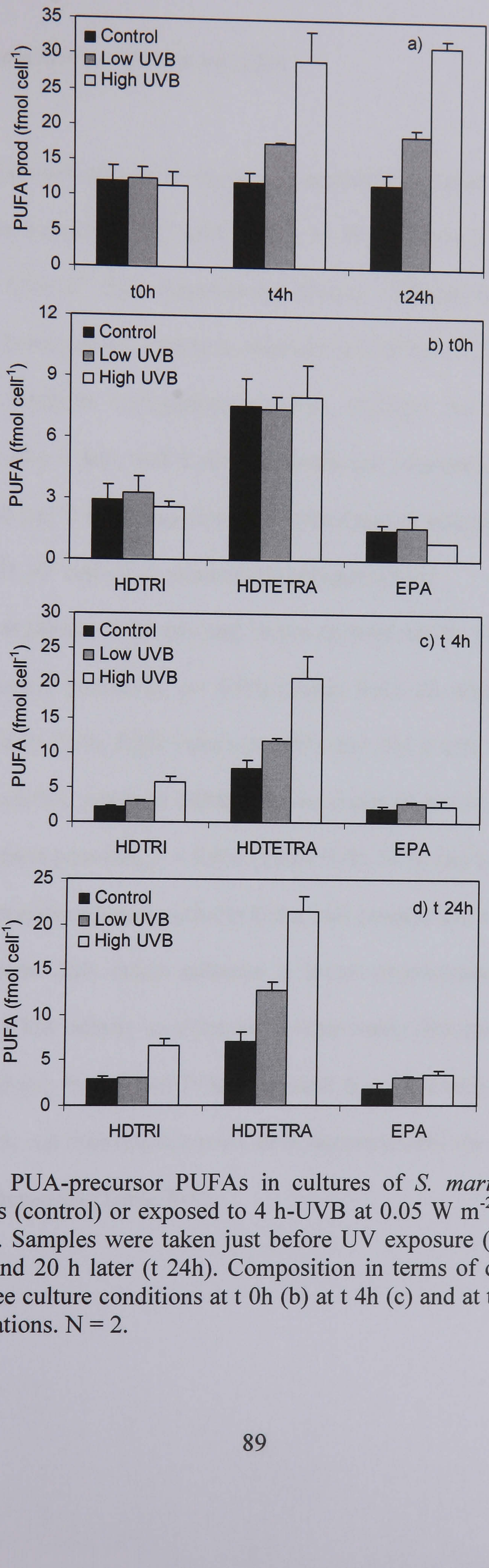


Figure II-15. (a) PUA-precursor PUFAs in cultures of *S. marinoi* under standard growth conditions (control) or exposed to 4 h-UVB at 0.05 W m^{-2} s^{-1} (Low) and 0.15 W m^{-2} s^{-1} (High). Samples were taken just before UV exposure (t 0h), just after UV exposure (t 4h) and 20 h later (t 24h). Composition in terms of different PUFA cell content at the three culture conditions at t 0h (b) at t 4h (c) and at t 24h (d). Error bars are standard deviations. N = 2.

II.3.6 PUA production at different salinities

The average growth rate of *S. marinoi* in exponential phase was not significantly different between normal salinity and salinity of 26 psu (with 0.97 d^{-1} ± 0.06 d^{-1} SD and 0.91 d^{-1} ± 0.02 d^{-1} SD, respectively) (Tukey Multiple Comparison post-test, $p>0.05$) while a lower growth rate was observed at salinity of 16 psu (0.77 d^{-1} ± 0.10 d^{-1} SD) (Tukey Multiple Comparison post-test, $p<0.05$). All cultures attained the stationary phase after 5 days with lower maximum cell concentrations at salinity of 16 psu (0.72 10^6 cell mL^{-1}) with respect to the control and at salinity of 26 psu (1.16 10^6 cell mL^{-1} and 1.03 10^6 cell mL^{-1}, respectively) (Figure II-16).

Cells grown at salinity of 26 psu and 16 psu showed similar FALS and RALS with respect to the control (ANOVA, $p> 0.05$) (Table II-8). At salinity of 16 psu, POC content decreased by 35%, RED values by 59% and Chl *a* cell content by 60% with respect with the control, while no difference was observed at salinity of 26 psu (Tukey Multiple Comparison post-test, $p > 0.05$) (Table II-8). Note that the decrease observed in the red fluorescence matched with the Chl *a* cell content and was consistent with a lower POC content. This result indicates a lower photosynthetic activity in cells acclimated to very low salinity as observed in other marin diatoms (see Kirst 1990). In all culture conditions, RALS and POC increased from mid-exponential to declining phase while FALS, red fluorescence and Chl *a* increased until the stationary phase and then decreased afterwards (Table II-8).

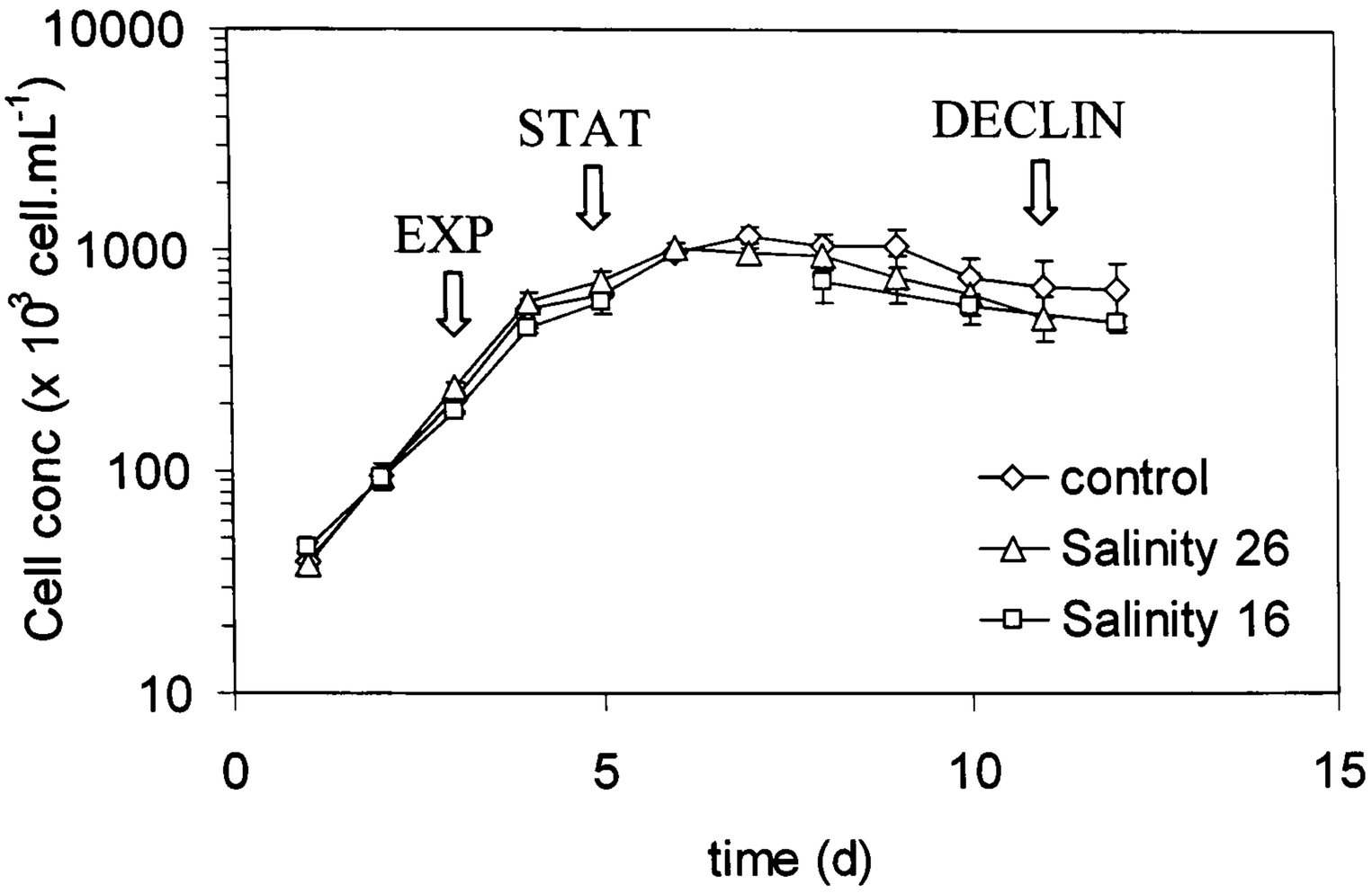


Figure II-16. Average cell concentrations of *S. marinoi* in batch cultures grown at salinities of 36.5 psu (control, open diamonds), 26 psu (open triangles) and 16 psu (open squares). Arrows indicate sampling points for analyses of polyunsaturated aldehydes (PUAs). Error bars are standard deviations (n = 4 for control experiment, n = 3 for lower salinity experiments)

Table II-8. Values of Forward Angle Light Scatter (FALS), Right Angle Light Scatter (RALS), red fluorescence from chlorophyll (RED), all expressed in units relative to the beads used as internal standard (r. u.), chlorophyll *a* (Chl *a*), particulate organic carbon (POC), and percentage of SYTOX Green positive cells (SYTOX+, non-viable) of *S. marinoi* under salinity of 36.5 psu (used as control), or salinities of 26 and 16 psu in exponential (EXP), stationary (STAT) and declining (DECLIN) phase of growth. Data are means ± standard deviations (n = 4 for control experiment, n = 3 for low salinity experiment).

Growth phase	Growth conditions	FALS (r. u.)	RALS (r. u.)	RED (r. u.)	Chl *a* (pg cell^{-1})	POC (pg cell^{-1})	SYTOX+ (%)
EXP	control	4.97 ± 0.55	0.57 ± 0.03	71.48 ± 11.73	0.74 ± 0.08	11.15 ± 1.22	0.7 ± 0.1
	Salinity 26	5.09 ± 0.98	0.57 ± 0.01	75.00 ± 1.73	0.74 ± 0.06	10.92 ± 3.11	0.6 ± 0.1
	Salinity 16	4.41 ± 0.23	0.59 ± 0.06	25.47 ± 1.22	0.41 ± 0.14	7.62 ± 0.45	2.8 ± 1.0
STAT	control	5.50 ± 0.24	0.68 ± 0.13	126.22 ± 21.75	1.25 ± 0.32	15.52 ± 2.78	2.1 ± 1.1
	Salinity 26	5.84 ± 0.37	0.68 ± 0.03	126.14 ± 2.23	1.10 ± 0.20	13.74 ± 0.92	0.7 ± 0.2
	Salinity 16	6.10 ± 0.27	0.78 ± 0.02	52.80 ± 7.44	0.54 ± 0.11	8.88 ± 0.75	3.8 ± 0.2
DECLIN	control	4.68 ± 0.80	1.15 ± 0.39	73.68 ± 19.73	1.25 ± 0.06	16.33 ± 2.94	15.8 ± 0.8
	Salinity 26	5.39 ± 0.31	2.23 ± 0.17	85.79 ± 3.35	0.98 ± 0.40	15.07 ± 1.35	13.8 ± 2.5
	Salinity 16	5.46 ± 0.47	1.18 ± 0.05	34.25 ± 1.14	0.27 ± 0.03	11.45 ± 1.09	24.6 ± 19.4

The proportion of SYTOX+ cells was very low in the exponential phase and stationary phase (>4%) and increased in declining phase to 15.8% (± 0.8% SD), 13.8% (± 2.5% SD) and 24.6% (± 19.6% SD) in the control and the cultures grown at salinities of 26 and 16, respectively (Table II-8). Difference among the different cultures were not significant (ANOVA, $p > 0.2$)

No significant change in PUA production was observed among the different salinities at the different growth phases (ANOVA, $p > 0.1$) (Figure II-17). Total PUA content per cell in the exponential phase of growth reached on average1.49 fmol cell^{-1} ± 0.19 fmol cell^{-1} SD and increased in the early stationary phase (3.66 fmol cell^{-1} ± 0.65 fmol cell^{-1} SD), remaining stable until the early declining phase (4.09 fmol cell^{-1} ± 0.50 fmol cell^{-1} SD, Figure II-17). The amount of 2,4-heptadienal, 2,4-octadienal and 2,4,7-octatrienal was not affected by a decrease in the salinity (ANOVA, $p > 0.1$) (Figure II-17b, c and d). Since POC and Chl *a* per cell was lower in cultures acclimated to salinity of 16 psu, PUA production normalized by POC or by Chl *a* content was higher with respect to the control (data not shown).

Since the PUA production was not affected by the salinity, the quantification of PUFAs was not performed.

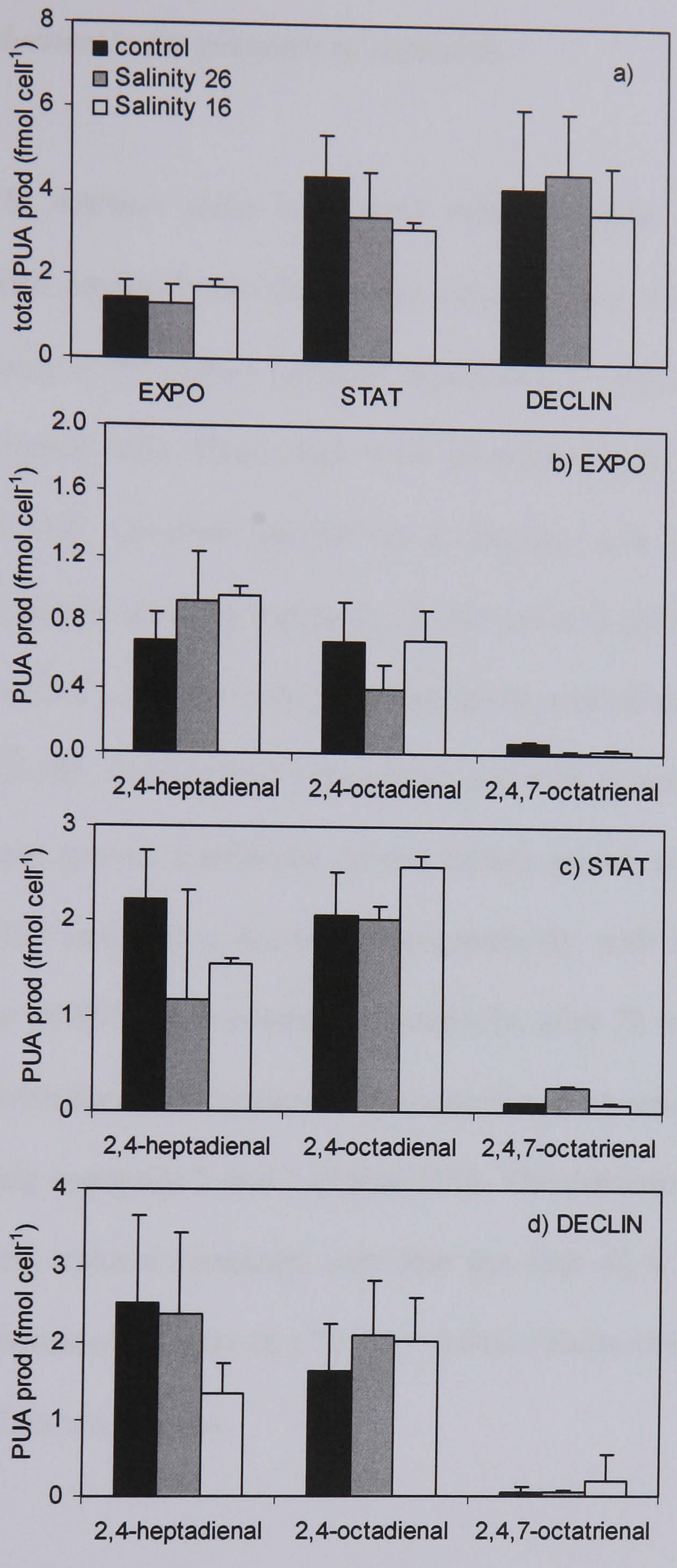


Figure II-17. (a) PUA production in cultures of *S. marinoi* grown at salinity of 36.5 psu (control) or at salinities of 26 and 16 psu. Samples were taken in exponential (EXP), stationary (STAT) and declining (DECLIN) phase of growth. Composition in terms of different PUFA cell content at the three culture conditions in exponential (b), stationary (c) and declining phase of growth (d). Error bars are standard deviations. (n = 4 for control experiment, n = 3 for lower salinity experiments).

II.3.7 PUA production in the presence of copepods

Cultures of *S. marinoi* were inoculated with copepods under three different treatments: (1) with copepods pre-fed on the dinoflagellate *Prorocentrum minimum* and inoculated freely in the culture (grazing copepods); (2) with pre-starved copepods put in tubes equipped with filters that were inoculated into the culture (starving copepods 1); (3) with copepods pre-fed on *S. marinoi* and put in a dialysis tube inoculated in the culture (starving copepods 2). All showed similar growth rates (1.18 d^{-1}, 1.08 d^{-1} and 1.10 d^{-1}, respectively) with respect to control cultures (1.10 d^{-1} ±0.05 d^{-1} SD) (Figure II-18). Cell optical parameters and Chl *a* content were differently affected in the three growth conditions. In the culture grown with grazing copepods, FALS, RALS, RED and Chl *a* decreased progressively with time to reach 60.4%, 43.7%, 50.2% and 56.9% of the control, respectively, after 72 h-inoculation, whereas an unclear effect with time with respect to the control was observed in the two cultures grown with starving copepods 1 and 2 (Table II-9). The percentage of SYTOX+ cells in the three treated cultures remained very low the first 48 h (< 2.1% of the total population) and increased slightly at t 72 h (< 6.0%) (Table II-9) with respect to the control (>3.5%) (data not shown).

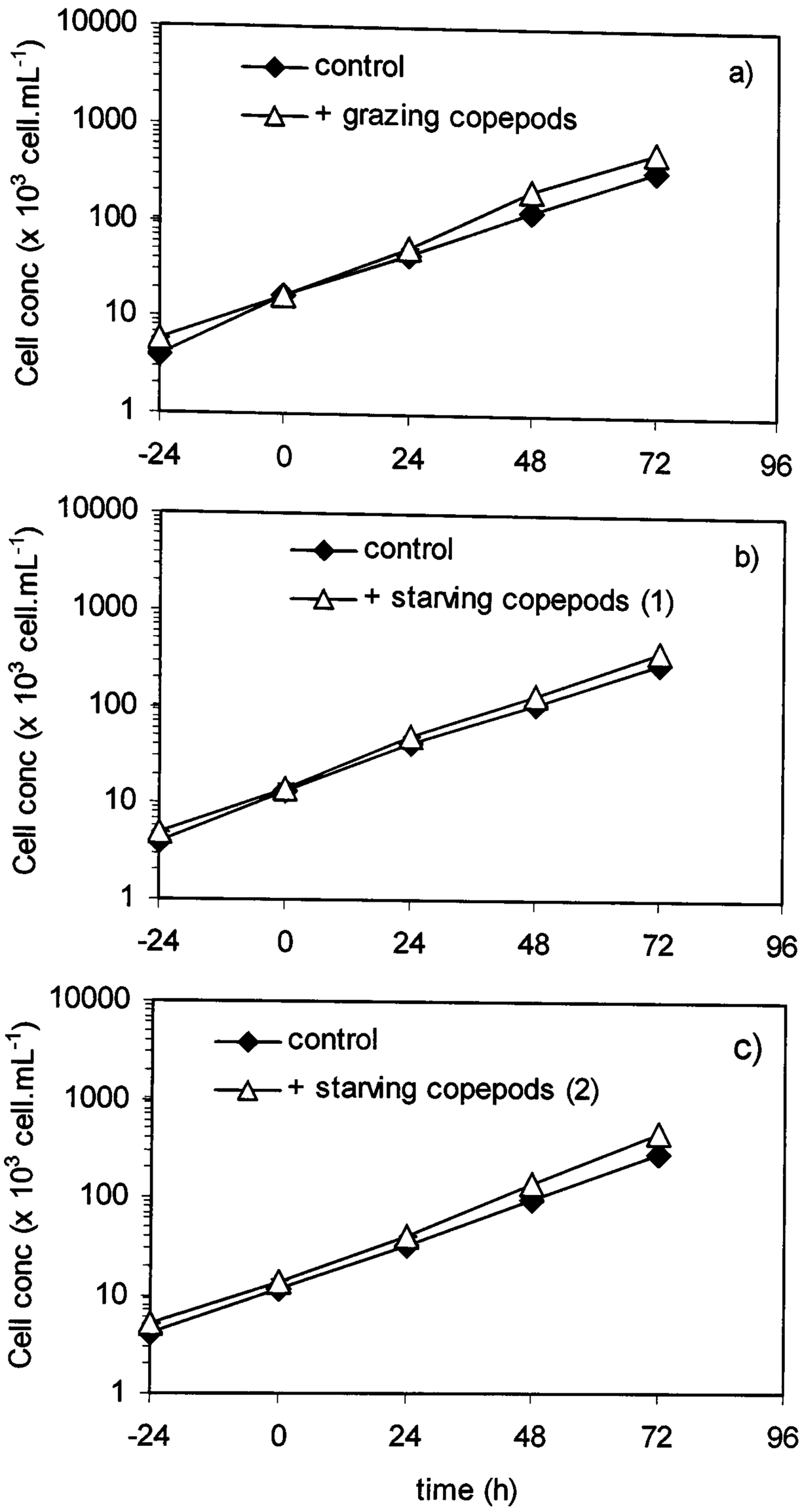


Figure II-18. Cell concentration of *S. marinoi* grown in 10-L batch culture with copepods (*Calanus helgolandicus*) or in a copepod-free growth medium (control). Arrow indicates when copepods were inoculated in the medium a) Copepods were pre-fed on the dinoflagellate *Prorocentrum minimum* and then introduced freely in the culture (+ grazing copepods). b) Copepods were starved and then inoculated in the culture into 5 Falcon tubes equipped with 0.22 µm Steriflip filters (+ starving copepods 1). c) Copepods were pre-fed on *S. marinoi* and then inoculated in a dialysis tubing cellulose membrane (+ starving copepods 2). Samples were taken at t 0h, 24h, 48h and 72h after copepod inoculation. A single replicate was performed for each growth condition.

Table II-9. Relative values of Forward Angle Light Scatter (FALS), Right Angle Light Scatter (RALS), red fluorescence from chlorophyll (RED), chlorophyll *a* (Chl *a*), and percentage of SYTOX Green positive cells (SYTOX+, non-viable) of *S. marinoi* grown with copepods (*Calanus helgolandicus*) with respect to the control (grown in copepod-free growth medium) at t 0h, 24h, 48h and 72h after copepod inoculation. Copepods were pre-fed on the dinoflagellate *Prorocentrum minimum* and then introduced freely in culture (+ grazing copepods); or starved and then inoculated in tubes equipped with 0.22 μm Steriflip filter (+ starving copepods 1); or pre-fed on *S. marinoi* and then inoculated in a dialysis tubing cellulose membrane (+ starving copepods 2). All units are expressed in percentage relative to the control (except for SYTOX+ cells expressed in percentage of the population). A single replicate was performed for each growth condition.

Experiment	Time (h)	FALS	RALS	RED	Chl *a*	SYTOX+
+ grazing copepod	0	88.1	103.0	97.0	107.0	0.4
	24	76.2	82.2	74.7	78.0	0.6
	48	76.0	68.8	73.1	60.2	1.2
	72	60.4	43.7	50.2	56.9	5.1
+ starving copepod 1	0	89.1	117.3	117.6	80.7	0.6
	24	95.7	112.9	106.1	85.6	0.8
	48	91.6	104.1	94.6	105.6	0.9
	72	88.7	101.7	93.6	93.0	6.0
+ starving copepod 2	0	88.0	147.3	95.8	87.5	0.5
	24	99.3	93.4	94.8	93.5	0.5
	48	88.8	109.1	92.5	88.2	2.1
	72	86.1	107.8	89.9	101.6	4.7

No significant change in PUA production was observed in any of the growth conditions (ANOVA, $p > 0.7$) (Figure II-19a, b and c). In the experiment with grazing copepods, PUA production remained stable at t 0h and t 24 h (<0.55 fmol cell^{-1} for both treated and control cultures) and increased from t 48 h (0.76 and 0.75 fmol cell^{-1}, respectively) to t 72 h (0.94 and 1.04 fmol cell^{-1}, respectively) (Figure II-19a); in the experiments with starving copepods 1 and 2, an increase of PUA production with time was also observed (Figure II-19b and c), except at t 48 h in the experiment with starving copepods 2 (Figure II-19c). However, the increasing PUA production with time was considered statistically significant (Tukey Multiple Comparison post-test, $p < 0.05$).

Since PUA production was not affected by the presence of copepods, the quantification of PUFAs was not performed.

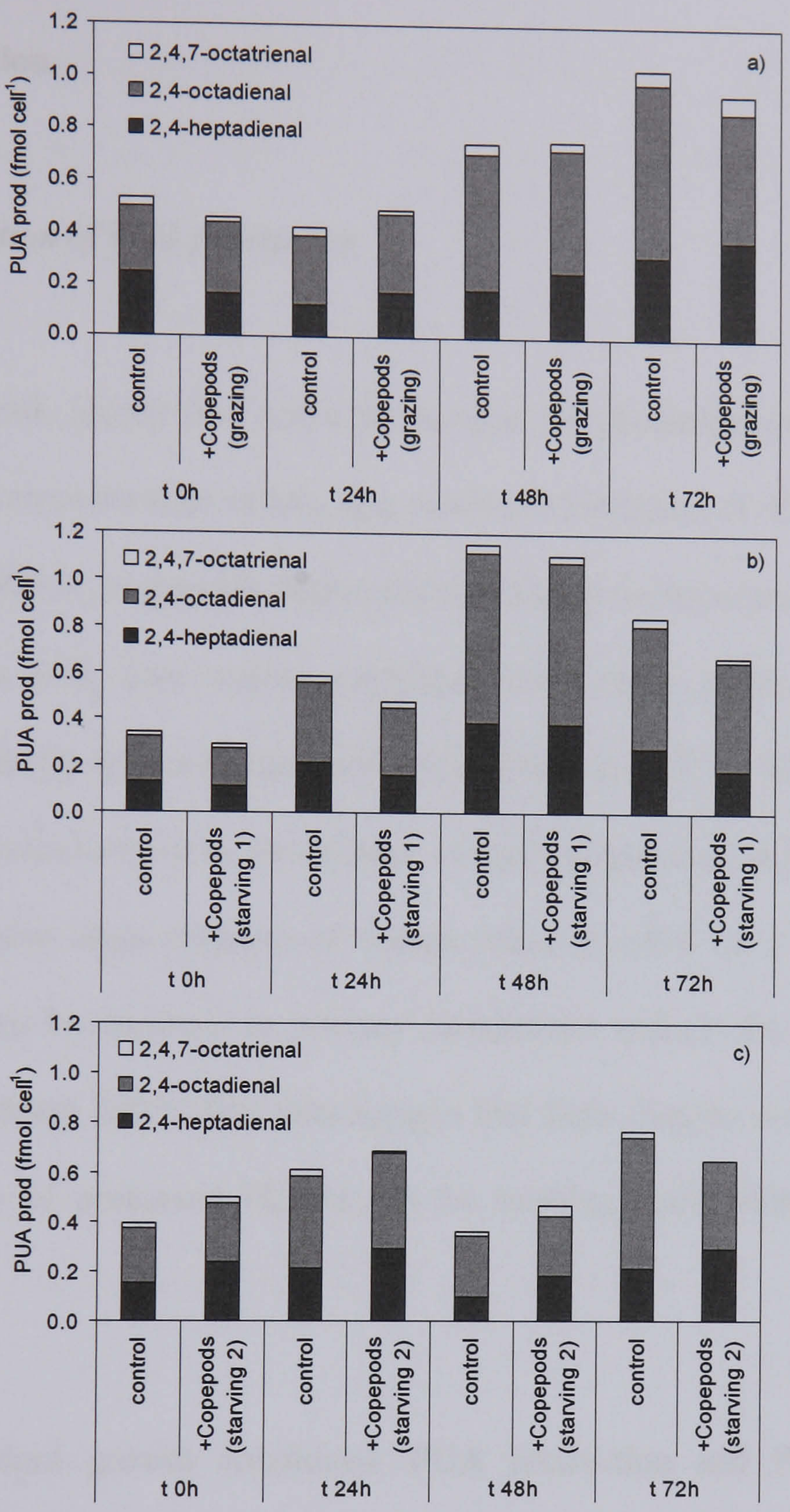


Figure II-19. Potential cell PUA production (2,4-heptadienal, 2,4-octadienal and 2,4,7-octatrienal) in *S. marinoi* grown in 10-L batch culture with copepods (*Calanus helgolandicus*) or in copepod-free growth medium (control). a) Copepods were pre-fed on the dinoflagellate *Prorocentrum minimum* (PM) and then introduced freely in the medium (+copepods, grazing). b) Copepods were starved and then inoculated in 5 tubes equipped with 0.22 µm Steriflip filters (+copepods, starving 1). c) Copepods were pre-fed on *S. marinoi* and then inoculated in a dialysis tubing cellulose membrane (+copepods, SM). Samples were taken at t 0h, 24h, 48h and 72h after copepod inoculation. A single replicate was performed for each growth condition.

II.4. Discussion

II.4.1 Modulation of PUA production

Previous work shows that toxin production by phytoplankton species is either constitutive or increases with culture age, related to limitation of different factors (e.g., Legrand et al. 2003). In general, these studies focused on structurally complex toxins, such a domoic acid, that require pathways involving a multitude of specialized enzymes and energy-rich cofactors such as NADPH or ATP for their biosynthesis. In contrast, the wound-activated production of polyunsaturated aldehydes in diatoms should not require large amounts of energy, since it relies on a few key enzymes, which could also be involved in primary metabolism and on the availability of free PUFAs (see Pohnert 2005). Our data suggest that both enzyme expression or activity and availability of precursor PUFAs can be limiting under different experimental conditions.

Under standard growth conditions, PUA production and PUFA cell content increased as the culture entered the stationary phase of growth. Berges et al. (2001) predict that phosphate should be the first nutrient to be depleted in the f/2 medium, which could consequently trigger a drop in EPA per diatom cell (Harrison et al. 1990). A depletion of phosphate is unlikely in the present study, since only addition of silicic acid and a mix silicic acid/nitrate in control cultures at the onset of the stationary phase initiates or triggers an increase in cell concentration, suggesting that silicic acid was

the main factor limiting growth, as reported also for the diatom *Pseudo-nitzschia multiseries* (Bates et al. 1989; Pan et al. 1996). Both polar and neutral lipids have been observed to increase in diatoms under nitrate and silicic acid deficiency, suggesting that deficiency of these two nutrients does not affect lipid synthesis directly, but reduces intracellular energy demand on storage lipids by causing cell divisions to stop (Roessler 1988; Dempster and Sommerfeld 1998). Therefore, it is likely that the depletion in silicic acid is responsible for the increase in PUA production observed in the early stationary phase under standard growth conditions.

In *S. marinoi,* more than 95% of the relevant PUA-precursor fatty acids are bound in phospho- and galactolipids (Berge et al. 1995) and would thus be available to the lipases releasing free fatty acids as substrates for processing to PUAs (Pohnert 2002; d'Ippolito et al. 2004). Even given a high variability in triacylglycerol content (1.7 – 14% of total lipids) in *S. marinoi* (Berge et al. 1995 and citations therein), it can be excluded that PUA-precursor fatty acids were predominantly bound to these lipids and were thus not available to the relevant enzymes. In the nutrient-replete cultures, it is unlikely that PUA synthesis was limited by PUFA cell content, since between 5- and 10-fold excess PUFAs were available in the total fatty acid pool, depending on the growth phase. In addition, the relative increase of specific precursor fatty acids (e.g., eicosapentaenoic acid) does not show a parallel increase of the corresponding PUAs (e.g., heptadienal) derived from these acids (Figure II-5 and Figure II-6). A possible explanation for this might be that, with the age of the culture, either PUFA release or their further transformation was prevented due to insufficiently active lipases or

lipoxygenases. It can thus be concluded that it is not the precursor availability but rather the enzyme activity that controls the absolute and the relative amount of PUA produced during normal growth conditions.

In the case of N-limitation (experiments with continuous cultures acclimated to low growth rate), the PUFA pool is depleted and low levels of PUAs are produced. Measurements of physiological indicators of nutrient-limitation, such as decreasing PON and Chl *a* concentrations and increasing C:N ratio in the case of N-limitation, point to cells experiencing increasing nutrient limitation with decreasing dilution rate (Sarthou et al. 2005; Wilhelm et al. 2006). When N-limited cells of the continuous cultures are transferred into batch cultures, and so are relieved of N-limitation, PON content increases together with PUA production (Figure II-8 and Table II-5). This may indicate that low levels of PON per cell observed in N-limited cultures (0.77 pg PON $cell^{-1}$, see Table II-3), may be critical for the production of PUAs in *S. marinoi* probably by limiting the synthesis of polypeptides and thereby enzymatic activities.

In the case of P- and Si-limitation, the level of PON is above this critical level (Table II-3) and PUAs are produced at a molar ratio of 2,4-heptadienal/eicosapentaenoic acid and 2,4-octadienal/hexadecatrienoic acid of 1:1 (Figure II-5 and 6). This suggests that under P- and Si-limitation most PUFAs are accessible for the phospho- and galactolipases initiating the transformations. It has been shown that gene expression of lipoxygenases is up-regulated under P-limitation in the coccolithophorid *Emiliania huxleyi* (Quinn et al. 2006), suggesting that a larger pool of active enzymes may also be available for PUA production in P- and Si-limited

diatoms. Thus, since the enzyme activity is greatly enhanced, cell PUA production under P- and Si-limitation might be limited by the amount of PUFAs available.

Irradiance can also affect PUA production and PUFA content. Indeed, under low photosynthetically active ration, cell PUA production does not increase with culture age and therefore is lower in stationary and declining cell phase with respect to higher light conditions (Figure II-11). The relative amount of precursor PUFA is also reduced but remains at least two times high as the PUA produced (Figure II-12). Assuming that the relevant PUFAs are bound to phospho- and galactolipids, this result suggests that PUA production is controlled by enzymatic activity rather by PUFA availability. Since PUA production does not respond to culture age under low light condition, suggests that photosynthetic energy requirements for PUA synthesis though a wound-activated mechanism should be an energy-independant process. Low photosynthetically active radiation induces a reduction of the electron transfer between photosystems I and II and thus impedes the formation of ATP and NADPH (Post et al. 1985) thereby reducing PUA biosynthesis. Low light could also have an indirect influence on PUA production since the uptake of nitrate is reduced leading to lower amounts of proteins in diatoms (Vergara et al. 1998), with priority given for chlorophyll synthesis and enzymes of the carbon dioxide reduction cycle (Anning et al. 2000). These effects of light on nitrogen metabolism could thus limit the supply of nitrogen required for PUA production, as observed under nutrient limitation. These findings suggest therefore that light can exert indirect effects on PUA production by controlling the enzyme activity involved in PUA production.

Under UVB exposure, PUA production and PUFA content increased together with UVB flux (Figure II-14). Here again, the level of each precursor PUFA exceeds by at least two-fold the amount of relative PUAs produced (Figure II-15), and thereby making the control of PUA production by enzyme activity most likely. The observation of a direct effect of UVB radiation on the PUFA content in *S. marinoi* may be due to the strong inhibition of cell division that leads to accumulation of photosynthetic products, as found in other marine diatoms (Leu et al. 2007). UVB radiation is also known to affect nutrient uptake and thereby may induce nutrient stress (Hessen et al. 1997). Therefore, since nutrient limitation affects the amount of PUAs produced, it is possible that nutrient stress induced by UVB exposure may contribute to the increase of PUA production. In addition, it has been recently shown that UVC radiation induces overexpression of lipoxygenase genes in plants (Bonomelli et al. 2004), which support the hypothesis of an enhanced enzyme activity of the lipoxygenase pathway following UV exposure in diatoms.

Since *S. marinoi* blooms occur in coastal areas, which can be exposed to a wide range of salinity caused by fluctuating river water inputs, as observed in the Northern Adriatic Sea (e. g. Pugnetti et al. 2004), the effect of salinity differences in PUA production has been assessed. No change in PUA production was observed within the range of salinities tested (16- 36.5 psu). Changes in the cell optical properties, Chl *a* and POC content were observed with decreasing salinity, while growth rates and PUA production remained similar. This indicates that *S. marinoi* cells may undergo morphological and physiological acclimation in withstanding the mixing of marine

and river waters in estuaries, which do not affect the amount of PUAs produced. A salinity of few psu decreases the growth rate of *S. marinoi* (Wiebe Kooistra, Stazione Zoologica di Napoli, unpublished data), and since changing PUA production appears to be associated with decreasing growth rate, it is not ruled out that the level of PUAs may be affected in this case. However, blooms of *S. marinoi* do not occur at such salinities, therefore this scenario is quite unlikely.

However, our experiments were performed on acclimated cells, while in nature the exposure is "pulsed" and not chronic. In this case of acute shift, cells may experience osmotic stress and a modulation of PUA production can be expected. Preliminary experiments on non-acclimated cells exposed to low salinity showed an increase of PUA production (data not shown).

Since these compounds are used as a chemical defense against copepods, one may expect an increase of toxin production in the presence of grazers. PUA production has been measured in exponentially growing cultures of *S. marinoi* in the presence of the co-occurring copepod, *Calanus helgolandicus*, but no effect was observed. A previous study shows that toxin production in the dinoflagellate *Alexandrium minutum* increases with increasing grazing copepod concentration from 4 to 16 individuals per liter, indicating that the algae was able to sense and respond to the presence of grazers (Selander et al. 2006). Since the concentration of 5 copepods per liter was used in our experiments, which represents "realistic" concentrations during blooms of *S. marinoi* (Adrianna Ianora, pers. comm.), it is possible that a higher concentration of copepods may be sensed by *S. marinoi* and would have lead to a modulation of PUA production.

In addition, it can not be excluded that with a different experimental set up, such as in the stationary phase where high cell density and no growth, so no possibility of replacement of cells, and consequently a higher need for defense may be required, a modulation of PUA production may occur.

It is well known that the environmental conditions under which phytoplankton grow and the growth stage in batch culture influence cellular chemical composition (Harrison et al. 1990). It is therefore not surprising that the toxin content of phytoplankton cells also varies with growth condition and stage in culture. The pattern of toxin production generally followed by dinoflagellates, for example, is that cellular levels are highest during the exponential phase and decrease during the stationary phase of growth (e. g. Anderson et al. 1990). This modulation of toxin production with the growth stage is markedly different from the pattern observed for PUA production in batch cultures of *S. marinoi*, in that toxin production increased only in the stationary phase. Indeed, the modulation of PUA production is very comparable to that of diatom-derived domoic acid produced in higher quantity during the stationary phase and under nutrient limitation (see Pan et al. 1998). Despite the fact that the mechanism of PUAs and domoic acid production differs substantially, changing environmental factors have similar effects: the production increases under silicon and phosphorus limitation (Fehling et al. 2004) and UV exposure (Bates et al. 1998), and decreases under nitrogen limitation (Bates et al. 1993) and low photosynthetically active radiations (Bates et al. 1991).

Chrysophytes in freshwater ecosystems, which produce similar PUAs, show species-specific responses in quantity and quality of PUAs produced as a function of growth phase, nutrient conditions and photosynthetically active radiation (Watson and Satchwill 2003). And also in chrysophytes no clear link has been observed between PUAs and PUFA content, implying that lipid composition and/or enzyme specificity are both important in PUA chemistry. It is interesting to note that PUA production in both freshwater chrysophytes and marine diatoms is modulated by nutrient stress and irradiance, suggesting a common mechanism controlling lipid metabolism.

II.4.2 Ecological impact

The variation of potential for PUA production with age of the culture suggests the endogenous and/or environmental control of diatom toxicity. Culture and bloom phase are thus very important when assessing the effect of a diatom diet upon copepod fitness or reproduction. PUAs have been shown to have teratogenic effects on grazers by inducing abortions, malformations and reduced larval growth (Ianora et al. 2004). In a broader survey of different diatom species and isolates in the stationary phase under standard growth conditions, PUA levels ranging from 0.01 fmol $cell^{-1}$ to 9.81 fmol $cell^{-1}$ have been observed in ca. 30% of the investigated diatoms, while 70% lacked these oxylipins (Wichard et al. 2005a). *S. marinoi*, which releases 4.21 fmol $cell^{-1}$ total PUAs during the stationary phase under similar growth conditions, thus is among the greatest producers so far investigated. In the laboratory, exponentially

growing cultures are most often used to test for possible teratogenic effects of diatom cells (Poulet et al. 1994). Our results suggest that these previous experiments may therefore have underestimated the impact of diatom PUAs on copepod reproduction, and that the potential number of species that have deleterious effects may be higher.

Caution should be taken when PUA production in nature is inferred from culture data as the production may change quickly depending on the growth phase (as observed between mid-exponential and early-stationary phase). Laboratory experiments should be consequently carefully designed to avoid an interfering influence of strongly fluctuating levels of PUA.

There is convincing evidence that the strong silicified cell wall of diatoms provides a quite effective mechanical protection against herbivore activity (Hamm et al. 2003), which could also be modulated by grazing pressure (Pondaven et al. 2007). However, silicified walls only prevent partly grazing on diatoms (Smetacek 1998) and do not prevent attacks by pathogens, such as parasites and viruses (Raven and Waite 2004; Nagasaki et al. 2004, 2005; Bettarel et al. 2005). During the evolution of a bloom at sea, phytoplankton species are thought to also utilize chemical signals or defense metabolites to enhance their resistance against grazers and pathogens (Landsberg 2002). This holds especially true at peak cell concentrations, when the probability of being grazed or infected increases. Interestingly, highest PUA production has been so far observed in some of the most common bloom-forming genera like *Thalassiosira* or *Skeletonema* (Wichard et al. 2005a). Several studies have invoked the role of a chemical defense in diatoms based on the production of PUAs to explain the observed

impairment of copepod reproduction during blooms (e. g. Miralto et al. 1999, Ianora et al. 2004). The modulation of PUA production with age of the culture supports the hypothesis of a direct link between toxin production and cell physiological state that is related to the level and type of nutrient limitation. This implies that diatom cells at the end of the bloom are better defended chemically against grazers than at the beginning of a bloom, and consequently grazers feeding at the end of a bloom would be more affected than early-bloom grazers. If silicic acid is the growth limiting factor at the end of diatom blooms, the barrier against grazers provided by the frustule may be weakened and thereby the diatom cells more vulnerable to grazers and pathogens. The great rise of PUA production induced by silicon limitation could represent an compensatory response of diatoms against grazers and pathogens. In this case, diatoms may have the possibility to switch from mechanical to chemical defense.

In addition, since diatom blooms occur in the first few meters below the sea surface and thereby are more exposed to UVB radiation, it is most likely that levels of PUA produced increase also during the early stage of the blooms and thereby enhancing the toxicity of diatoms on copepod reproduction, as observed in *Calanus helgolandicus* feeding on UVB-exposed *Skeletonema costatum* (Kouwenberg and Lantoine 2007).

So far, most of the environmental factors tested that induce an acute stress stimulate PUA production. A decrease of PUA production has been observed only in low-light acclimated cells. However, diatom blooms such as those of *Skeletonema*,

usually occur when light is not limiting (Hitchcock and Smayda 1977) and therefore an decreased of PUA production by low light is an unlikely scenario.

II.5. Conclusions

A strong dependence of PUA production on culture age, nutrient limitation and other stress factors, such as UV exposure and low light conditions, has been observed in cultures of *S. marinoi*, suggesting a direct link between toxin production and cell physiological state. Essential nutrients, when limiting, may decrease primary metabolism thereby making available necessary precursor fatty acids, and/or enhance enzymatic activity of the lipoxygenase pathway by inducing gene expression of the necessary enzymes. This has direct implications for natural conditions, since nutrient limitation is common and usually the factor triggering the end of a bloom at sea (Cullen 1991). Due to human activities that increase inputs of nutrients, the N:P:Si ratio has changed considerably in the surface seawaters of many coastal areas, where most algal blooms occur. This in turn may impact diatom physiology by strongly affecting the production of PUAs and enhancing their impact on the trophic food web.

The involvement of enzymes, such as lipases and lipoxygenase, and their kinetics deserves therefore further investigation in order to better understand the effects of various environmental stresses. In addition, the identification of genes responsible for PUA biosynthesis will be very useful to design molecular probes to determine quickly which forms and species of diatoms have the capacity to produce PUAs in nature.

CHAPTER 3 Toxicity of diatom-derived polyunsaturated aldehydes

I. Growth inhibition of cultured marine phytoplankton by toxic algal-derived polyunsaturated aldehydes

(Results presented here have been published in Ribalet, F., Berges, J.A., Ianora, A., and Casotti, R., 2007 Growth inhibition of cultured marine phytoplankton by toxic algal-derived polyunsaturated aldehydes. Aquatic Toxicology 85, 219-227)

I.1. Introduction

PUAs produced by phytoplankton include 2E,4E/Z-heptadienal, 2E,4E/Z-octadienal, 2E,4E/Z,7Z-octatrienal, 2E,4E/Z-decadienal and 2E,4E/Z,7Z-decatrienal, and these are described for both freshwater and marine phytoplankters, including chrysophytes, cryptophytes, cyanobacteria, synurophytes, prymnesiophytes and diatoms (Hansen et al. 2004a; Watson 2003; Watson and Satchwill 2003; Watson et al. 2001; Wichard et al. 2005a). Among these PUAs, 2E,4E-decadienal has been widely used as a model aldehyde and has shown to induce deleterious effects on the reproduction of several invertebrates, such as echinoderms, polychaetes, ascidians,

crustaceans and molluscs (Caldwell et al. 2003). However, little information is available on other PUAs and on their effect on photosynthetic organisms that coexist in natural phytoplankton communities. Casotti et al. (2005) have investigated the effect of this aldehyde on the diatom *Thalassiosira weissflogii* in culture and shown that it induced reduction of the growth rate and triggered programmed cell death. It has also been suggested that decadienal may play a role to activate a stress-signalling mechanism mediated by Ca^{2+} and nitric oxide in the diatom *Phaeodactylum tricornutum* (Vardi et al. 2006). For these effects to occur in nature, however, it is necessary that PUAs are released in the surrounding medium following cell breakage. So far, only mastication by grazers has been suggested as a possible trigger for PUA formation. However, cell lysis triggered by endogenous or exogenous factors such as senescence or viral attack, is also a possible and widespread phenomenon occurring at sea (Kirchman 1999). Once released in the water, PUAs are expected to interact with organisms living in the vicinity of PUA-producing cells, by influencing their growth performance. Therefore, it is reasonable to propose a role of these compounds as allelochemicals.

There is some evidence of such a role in nature. Recently, Yamakashi et al. (2007) observed that the phytoplankton community in Hakozaki harbor was alternatively dominated by blooms of the PUA-producing diatom *Skeletonema costatum* and the dinoflagellate *Heterosigma akashiwo*. These authors reported that the growth of the two species were reciprocally suppressed and hypothesized that both species may secrete allelochemicals. On the contrary, a recent study on the prymnesiophyte PUA-producing *Phaeocystis pouchetii* found no correlation between the presence of this

species and diatom diversity (Hansen and Eilertsen 2007), concluding that it is unlikely that PUA production can offer to the producer a competitive advantage against other phytoplankton species. However, both *Phaeocystis* and diatoms produce PUAs and often coexist at sea, and the possibility that other species are affected by PUAs released into seawater cannot be ruled out.

The aim of the present study is to investigate the effect of three different PUAs on six species of algae belonging to taxonomic groups which are well represented in marine areas. Fluorescent stains detected by flow cytometry and epifluorescence were used to assess the effect of PUA exposure on growth rate, cell membrane integrity and morphology in culture.

I.2. Materials and Methods

I.2.1 Algal cultures and experimental design

Axenic cultures of the diatom *Skeletonema marinoi* Sarno & Zingone (reallocated from *S. costatum*, strain CCMP2092 (Sarno et al. 2005)), the chlorophyte *Dunaliella tertiolecta* Butcher (strain CCMP1320), the prymnesiophyte *Isochrysis galbana* Parke (strain CCMP1323), the dinophyte *Amphidinium carterae* Hulburt (strain CCMP1314), the two prasinophytes *Tetraselmis suecica* (Kylin) Butcher (strain CCMP906) and *Micromonas pusilla* (Butcher) Manton & Parke (strain CCMP1646) were all obtained from the the Provasoli-Guillard National Center for Culture of

Marine Phytoplankton (Boothbay Harbor, USA). The cultures were grown semi-continuously at 15°C (except for *Micromonas pusilla* grown at 18°C) with a 12h -12h light-dark cycle under a photon flux density of 150 µmol m^{-2} s^{-1} provided by white fluorescent tubes (Philips TLD 36W/950). Filtered seawater from the Gulf of Naples was amended with f/2 nutrients (Guillard 1975) and used as medium. Cell growth was monitored by measuring cell numbers by flow cytometry. Growth rates were calculated as µ (d^{-1}) according to:

$$\mu(\mathrm{d}^{-1}) = \ln \frac{N_1 / N_0}{t} \qquad (1)$$

where N_0 and N_1 represent cell concentration at the start and the end of the growth period, and t the time between measurements (in days).

For the toxicity tests 50 ml of each exponentially growing culture were inoculated with different concentrations (from 0.1 to 36 µmol L^{-1}) of each of the three PUAs. Initial cell concentration and growth rates are reported in Table III-1. Experiments were replicated at least twice at different times, with three independent cultures for each PUA concentration in each replicate.

Table III-1. Species used in this study, their taxonomic affiliation, average cell volume (μm^3) and growth rates at exponential phase (d^{-1}). Data are means of duplicate cultures from 3 independent experiments, with standard deviations (n = 6). Cultures were grown in f/2 medium with a 12h -12h light-dark illumination under a photon flux density of 150 $\mu mol\ m^{-2}\ s^{-1}$.

Species	Taxonomic affiliation	Volume of control cells (μm^3)	Growth rate of control cells (d^{-1})
Micromonas pusilla (MP)	Prasinophyceae	4.7 ± 3.8	0.84 ± 0.05
Tetraselmis suecica (TS)	Prasinophyceae	1843 ± 362	0.96 ± 0.07
Isochrysis galbana (IG)	Prymnesiophyceae	26.3 ± 11.5	0.65 ± 0.03
Amphidinium carterae (AC)	Dinophyceae	2299 ± 635	0.43 ± 0.02
Dunaliella tertiolecta (DT)	Chlorophyceae	777 ± 276	0.69 ± 0.09
Skeletonema marinoi (SM)	Bacillariophyceae	63.4 ± 12.9	0.85 ± 0.08

I.2.2 PUA preparation

The PUAs 2E,4E-heptadienal, 2E,4E-octadienal, and 2E,4E-decadienal were obtained from Sigma-Aldrich Inc. (Milano, Italy). Working solutions of PUAs were prepared by diluting the stock in absolute methanol (ROMIL, Cambridge, UK) at room temperature. The effective PUA concentration of the working solution was assessed spectrophotometrically before inoculation at a wavelength of 274 nm using a specific molar absorption coefficient of 31000 (Pippen and Nonaka 1958). The methanol had no effect on growth up to 7 µL of 100% methanol per mL of culture (data not shown) and the amount of aldehyde solution in each test was kept well below this threshold.

I.2.3 Flow cytometry, cellular parameters and cell viability

A Becton-Dickinson FACScalibur flow cytometer equipped with an air-cooled 488 nm argon-ion laser was used to estimate cell concentrations, percentage of viable cells and cell inherent optical parameters (scatter and fluorescence). The sheath fluid was natural seawater filtered through 0.22 µm polycarbonate filters (Nuclepore, Pleasanton, USA). Optical properties of cells were assessed using Forward Angle Light Scatter (FALS) as a proxy of size and Right Angle Light Scatter (RALS), which is sensitive to the particle refractive index, as an indicator of changes in cell morphology that are termed "granulosity". Red fluorescence was collected through a

650 long-pass filter and was used as a proxy for cell chlorophyll content. All values were expressed as a ratio between treated cells and non-treated cells units relative to the beads used as internal standards (1 µm Polysciences fluorospheres, Warrington, USA, for *M. pusilla* and *I. galbana*, and 3.7 µm beads Coulter Flow-Set fluorospheres, Beckman-Coulter, Fullerton, USA for *S. marinoi*, *D. tertiolecta*, *A. carterae*, and *T. suecica*). Data acquisition (10^4 cells on average for each sample) and analysis were performed using CellQuest software (Becton-Dickinson, San José, USA).

The percentage of viable cells was assessed in samples exposed to twice the EC_{50} concentration (determined for each individual PUAs; see below) 24 h and 48 h after exposure. This concentration was chosen to compare species with different sensitivity to the PUAs at similar effect levels. Viability was assessed using the vital stain SYTOX Green (Molecular Probes, Leiden, The Nederlands) (Casotti et al. 2005). This stain does not penetrate live cells but only those with compromised plasma membranes. Optimal final concentration used was 500 nmol L^{-1} and time of incubation was 10 min. The green fluorescence of stained cells was collected through a 530/30-nm bandpass filter.

I.2.4 Cell and nuclear morphology

Morphological observations were obtained from at least 150 cells at 1000 x magnification using an Axioskop 2 microscope (Carl Zeiss GmbH, Jena, Germany)

equipped with transmitted and epifluorescence light under blue excitation. Nucleus morphology observations were obtained from cells fixed with 1% paraformaldehyde and stained with the green-fluorescing DNA stain SYBR Green I (Molecular Probes, Leiden, The Netherlands) for 10 minutes at room temperature in the dark. For *D. tertiolecta* it was_necessary to employ an antifading reagent (0.1% phenylenediamine, 50% glycerol and 50% PBS), made fresh daily, to prolong fluorescence (Noble and Fuhrman 1998). Samples were taken from cultures exposed to twice the EC_{50} concentration (determined for the individual PUA; see below) at 24 h and 48 h after exposure. This concentration was chosen to compare species with different sensitivity to the PUAs at similar effect levels.

Cell size was measured in living samples of control cultures (minimum 20 cells). Linear measurements were converted into cell volume using different geometric approximations: a sphere for *M. pusilla* and *I. galbana*, an ellipsoid for *A. carterae*, *D. tertiolecta* and *T. suecica*, and a cylinder for *S. marinoi* (Table III-1).

I.2.5 Statistical analyses

The acute toxicity of PUAs was expressed as EC_{50}, which is the concentration of PUA inducing a reduction of 50% in growth relative to the control, after 24 h exposure. EC_{50} values with 95% confidence interval were obtained from Probit analysis from a linear regression of percentage growth on logarithmic concentrations

of PUA (Newman 1995). Transformed Probit values (natural logarithm of arcsinus of the square root divided by 100) were used to obtain a normal distribution and equal variance to allow statistical comparison between treatments and species, using a Holm-Sidak Multiple Comparison post-test (SigmaStat 3.0, SPSS Inc., Chicago, USA).

Data were fitted by a "dose-response model" using a non linear regression with the following four-parameter logistic equation (Prism 4, GraphPad Software, San Diego, USA) which has been used before by Chèvre et al. (2002):

$$Y = bottom + (Top - Bottom) \times \left(1 + \left(\frac{10^{\log EC50}}{10^{X}}\right)^{slope}\right)^{-1} \quad (2)$$

where "*Bottom*" indicates the background value in the absence of PUA, "*Top*" is the value representing the response produced by the highest PUA concentration, $LogEC_{50}$, is the log of the PUA concentration that induces growth values situated halfway (50%) between *Bottom* and *Top*, and "*Slope*" is the slope of the curve, used as a measure of the responsiveness of the algal growth to increments in PUA concentrations. The same equation was used to assess the value of the highest PUA concentration for which no effect is observed as compared to the control, defined as the statistical no effect concentration (SNEC). The SNEC is based on the 95% confidence limit modeled by the non linear regression model. This parameter is preferred to the No Observed Effect Concentration (NOEC), because it is less variable and less dependent on the experimental design (Chèvre et al. 2002).

I.3. Results

I.3.1 Effect of PUAs on growth rate

A concentration-dependent decrease of growth rate in cultures was observed for all cultures and for all PUAs, with 2E,4E-decadienal inducing a stronger effect than for the other polyunsaturated aldehydes (Holm-Sidak Multiple Comparison post-test $p<0.05$) (Figure III-1). When comparing SNEC values it is evident that the six species responded differently to the three PUAs (Table III-2). The growth rate of *I. galbana* was significantly reduced with 2E,4E-decadienal concentrations above 0.10 µmol L^{-1} (95% CI: 0.08-0.13µmol L^{-1}) while significant growth inhibition in *S. marinoi* started at concentrations only twenty times higher (2.20 µmol L^{-1} with 95% CI: 1.94-2.45 µmol L^{-1}) (Table III-2). Note that at concentration of 0.8 and 0.9 µmol L-1 of 2E,4E-heptadienal and 2,4-octadienal the growth rate of *S. marinoi* was slightly stimulated (Figure III-1f). Except for *M. pusilla*, for which the SNEC for 2E,4E-octadienal was lower than the two other PUAs, the SNEC values for 2E,4E-octadienal and 2E,4E-heptadienal were higher than those for 2E,4E-decadienal. For *I. galbana*, these values were nineteen and thirty times higher than those for 2E,4E-decadienal (1.86, 3.06 and 0.10 µmol L^{-1}, respectively, Table III-2).

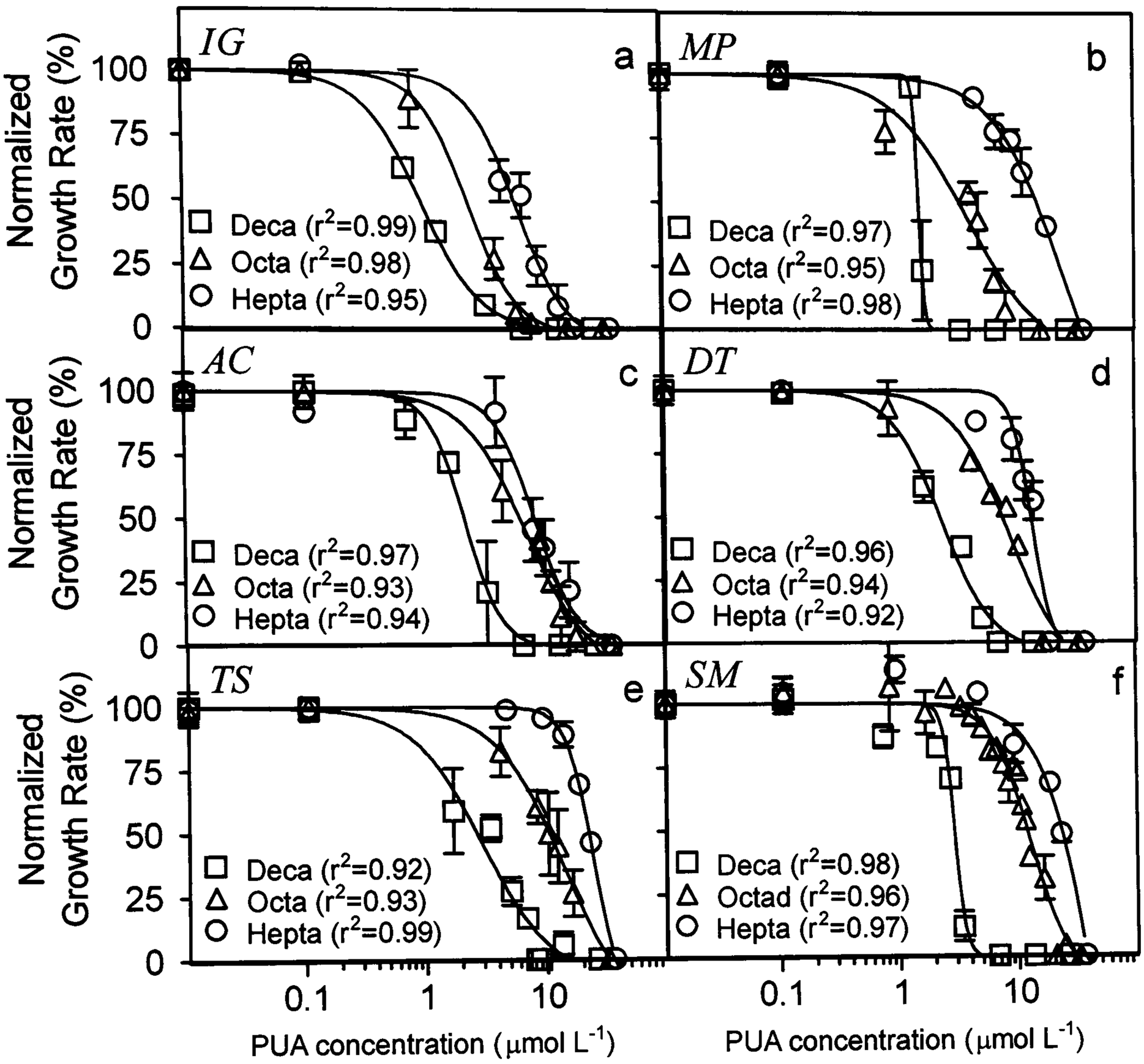


Figure III-1. Reduction in growth rates after 24h as a function of PUA concentration (µmol L^{-1}) of 2E,4E-decadienal (deca), 2E,4E-octadienal (octa) and 2E,4E-heptadienal (hepta) for a) *Isochrysis galbana* (IG), b) *Micromonas pusilla* (MP), c) *Amphidinium carterae* (AC), d) *Dunaliella tertiolecta* (DT), e) *Tetraselmis suecica* (TS) and f) *Skeletonema marinoi* (SM). Data are normalized by the growth rate of the control cultures (untreated) and fitted using a non-linear regression model. The correlation coefficient (r^2) for each curve is indicated on their respective panels. Data are means of replicates with standard deviations (n = 3).

Table III-2: Values of the Statistical No observed Effect Concentration (µmol L^{-1}), and of the 24 h-EC_{50} (µmol L^{-1}) of six species of marine phytoplankton to 2E,4E-decadienal, 2E,4E-octadienal and 2E,4E-heptadienal. EC_{50} values were estimated by Probit analysis; the Statistical No Effect Concentration was determined from the dose-response curve fitted by non-linear regression model at 4 parameters (see Methods). Note that the lower the values, the more sensitive the species. Data are means of replicates with 95% confidence intervals (n = 3). IG is for *Isochrysis galbana*, MP for *Micromonas pusilla*, AC for *Amphidinium carterae*, DT for *Dunaliella tertiolecta*, TS for *Tetraselmis suecica* and SM for *Skeletonema marinoi*.

Species	Statistical No Effect Concentration (µmol L^{-1})			24h-EC_{50} (µmol L^{-1})		
	Decadienal	Octadienal	Heptadienal	Decadienal	Octadienal	Heptadienal
IG	0.10 (0.08 -0.13)	1.86 (1.41-2.32)	3.06 (1.33-4.79)	0.99 a (0.76-1.22)	2.25 a (1.68-2.91)	5.90 a (4.53-7.27)
MP	1.42 (1.30-1.54)	0.41 (0.03-0.79)	4.03 (2.14-5.92)	1.19 a (0.38-1.99)	3.24 b (2.39-4.08)	11.32 b (10.48-12.17)
AC	1.27 (1.04-1.50)	4.75 (2.52-6.98)	4.50 (1.61-7.38)	1.67 b (0.74-2.61)	5.27 c (3.15-7.39)	8.95 b (8.41-9.49)
DT	1.20 (0.45-1.95)	4.03 (0.68-7.38)	12.11 (9.13-15.09)	2.17 b (1.63-2.71)	5.67 c (5.22-6.11)	10.71 b (8.84-12.57)
TS	1.50 (0.33-2.67)	5.30 (3.41-7.20)	12.79 (10.50-15.09)	2.02 b (1.71-2.32)	8.16 d (5.69-10.60)	15.57 c (13.15-17.99)
SM	2.20 (1.94-2.45)	3.23 (1.97-4.50)	3.61 (2.12-5.10)	2.48 b (1.81-3.14)	8.94 d (7.28-10.60)	18.17 c (16.76-19.59)

a, b, c and d : EC_{50} values with the same letter indicate that the difference between two species for the same PUA is not significant ($\underline{P}$>0.05). A multiple comparison procedure was applied for comparing the values between the different species, for each aldehyde (Holm-Sidak method, SigmaStat 3.0).

The acute toxicity of PUAs, estimated by the 24h-EC_{50} (which represents the PUA concentration inducing 50% growth inhibition), differed between species. *I. galbana* showed an EC_{50} of 0.99 µmol L^{-1} for 2E,4E-decadienal, 2.25 µmol L^{-1} for 2E,4E-octadienal and 5.90 µmol L^{-1} for 2E,4E-heptadienal. Values were 2.02, 8.16 and 15.57 µmol L^{-1} for *I. galbana* and *T. suecica*, while for *S. marinoi* the EC_{50} was 2.48, 8.94 and 18.17 µmol L^{-1} for the three PUAs, respectively. No significant difference was observed between *S. marinoi* and *T. suecica*, and between *A. carterae* and *D. tertiolecta* when the EC_{50} values for each PUA were compared (Student *t*-test, $p>0.05$).

Aside from variation in the effective concentrations between species, changes were also noted in the growth rate with increasing PUA concentrations. This was reflected in the slope of the growth rate *vs* concentration relationship (Figure III-1) which can be termed "responsiveness" (Table III-3). Values were very similar for most of the species and only slight differences were observed between the three PUAs. In the case of *S. marinoi* and *M. pusilla*, the growth rate was particularly responsive to a step-wise increase in 2E,4E-decadienal (slope coefficients of 9.54 and 16.28, respectively) as compared to 2E,4E-octadienal (slope coefficients of 2.61 and 1.92, respectively) and 2E,4E-heptadienal (slope coefficients of 1.35 and 1.50, respectively), while *T. suecica* and *D. tertiolecta* were slightly more responsive to 2E,4E-heptadienal (slope coefficients of 3.89 and 5.19, respectively) than the two other PUAs (slope coefficients of 1.50 and 2.01 for 2E,4E-decadienal, respectively; and 1.52 and 1.81 for 2E,4E-octadienal, respectively) (Table III-3).

Table III-3: Values of the equation coefficients expressing the slope of the dose-response curve, representing the responsiveness of the growth rate of a given species to an increment of PUA concentration, for the six phytoplankton species exposed to 2E,4E-decadienal, 2E,4E-octadienal and 2E,4E-heptadienal. Only absolute values are reported. Note that the higher the value, the more responsive the species to PUA. Data are means of replicates with 95% confidence interval limits (n = 3). *IG* is for *Isochrysis galbana*, *MP* for *Micromonas_pusilla*, *AC* for *Amphidinium carterae*, *DT* for *Dunaliella tertiolecta*, *TS* for *Tetraselmis_suecica* and *SM* for *Skeletonema marinoi*.

Species	Slope		
	Decadienal	Octadienal	Heptadienal
IG	1.75 (1.58-1.91)	2.15 (1.64-2.67)	2.06 (1.30-2.81)
MP	16.28 (8.56-20.56)	1.35 (0.81-1.90)	1.50 (1.08-1.94)
AC	2.87 (3.78-1.96)	1.68 (1.01-2.35)	2.50 (1.46-3.54)
DT	2.01 (1.43-2.60)	1.81 (1.06-2.57)	5.19 (2.91-7.46)
TS	1.50 (0.92-2.07)	1.52 (0.77-2.28)	3.89 (3.30-4.48)
SM	9.54 (6.50-12.58)	2.61 (1.88-3.33)	1.92 (0.58-3.26)

I.3.2 Effect of PUAs on inherent optical parameters and morphology

FALS, RALS and RED were affected after 24 h PUA exposure in all species, but in varying ways (Figure III-2). After 24 h at twice the EC_{50} value, cell size, estimated by the FALS value, was slightly reduced in most of the species, except for *M. pusilla* for which a strong decrease was observed (39.9 ± 24.5%, 63.9 ± 4.6%, and 75.3 ± 12.0%, for 2E,4E-decadienal, 2E,4E-octadienal and 2E,4E-heptadienal, respectively); and in the case of *I. galbana*, a slight increase of FALS was observed (105.8 ± 4.6 %, 112.7 ± 4.2 %, and 111.4 ± 0.6 %, for the three PUAs, respectively) (Figure III-2a). RALS, which is a parameter sensitive to changes in internal cell granulosity, increased in all species except *A. carterae* (Figure III-2b). The mean cell red fluorescence from chlorophyll decreased in all species. *A. carterae* fluorescence was the most affected, decreasing 41.4 ± 16.3 %, 59.6 ± 4.1 % and 59.7 ± 0.2 % of the control values for 2E,4E-decadienal, 2E,4E-octadienal and 2E,4E-heptadienal, respectively, indicating strong chlorophyll quenching as related to PUA toxicity (Figure III-2c). No different effect on flow cytometric properties was observed between the three PUAs.

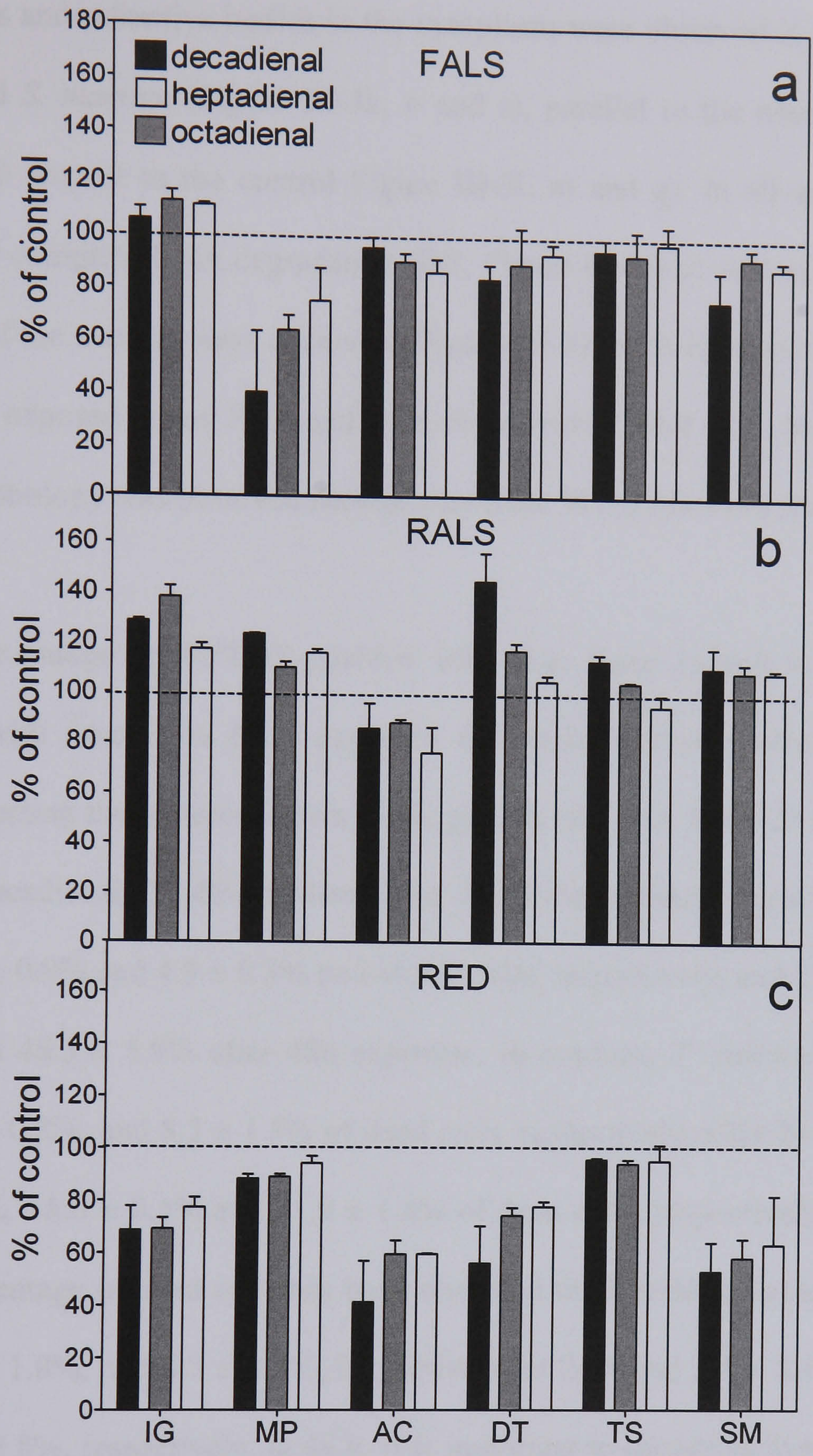


Figure III-2. Mean cellular values of a) FALS, b) RALS, and c) RED fluorescence of *Isochrysis galbana* (IG), *Micromonas pusilla* (MP), *Amphidinium carterae* (AC), *Dunaliella tertiolecta* (DT), *Tetraselmis suecica* (TS) and *Skeletonema marinoi* (SM) after 24 h of incubation with 2E,4E-decadienal, 2E,4E-octadienal, or 2E,4E-heptadienal, at twice the concentration of the individual EC_{50} values each. The dotted line indicates the 100% which is the reference value (control). Data are means of replicates with standard deviations (n = 3).

Vesicles and refractive bodies in the cytoplasm were observed in *D. tertiolecta*, *T. suecica* and *S. marinoi* (Figure III-3k, o and s), parallel to the observed increase in RALS, with respect to the control Figure III-3i, m and q). In all species chromatin dispersal or complete DNA degradation (DT, Figure III-3i) as well as modifications of the shape of the nucleus were observed (Figure III-3). Such changes were observed in all cultures exposed to any PUA and were most evident after 48 h. No different effect on the morphology was observed between the three PUAs (data not shown).

The percentage of SYTOX-positive cells (i.e. those judged to be non-viable) increased with time upon PUA exposure and varied within species (Figure III-4), largely reflecting the patterns shown in the growth rate data. After 24 h PUA exposure to 2E,4E-decadienal, 2E,4E-octadienal and 2E,4E-heptadienal, *I. galbana* had 15.2 ± 1.2 %, 8.2 ± 0.9% and 4.9 ± 0.3% non-viable cells, respectively, and 51.6 ± 2.6%, 42.6 ± 2.8% and 48.3 ± 5.9% after 48h exposure. In contrast, *T. suecica* had only 7.3 ± 1.8%, 8.0 ± 0.8%, and 8.3 ± 1.5% of dead cells, respectively, after 24 h exposure, and 15.9 ± 1.6%, 18.0 ± 0.4% and 21.9 ± 1.4% of dead cells, respectively, after 48h. The lowest percentage of dead cells has been observed in *D. tertiolecta* culture with 2.0 ± 0.2%, 3.0 ± 1.8%, and 4.3 ± 0.5%, respectively, at 24 h and 2.5 ± 2.4%, 12.0 ± 0.8% and 11.6 ± 3.8%, respectively, at 48 h. It is important to recognize that staining results may have been underestimated in *D. tertiolecta* because of the high level of chromatin degradation (Figure III-3o) which may have interfered with SYTOX staining.

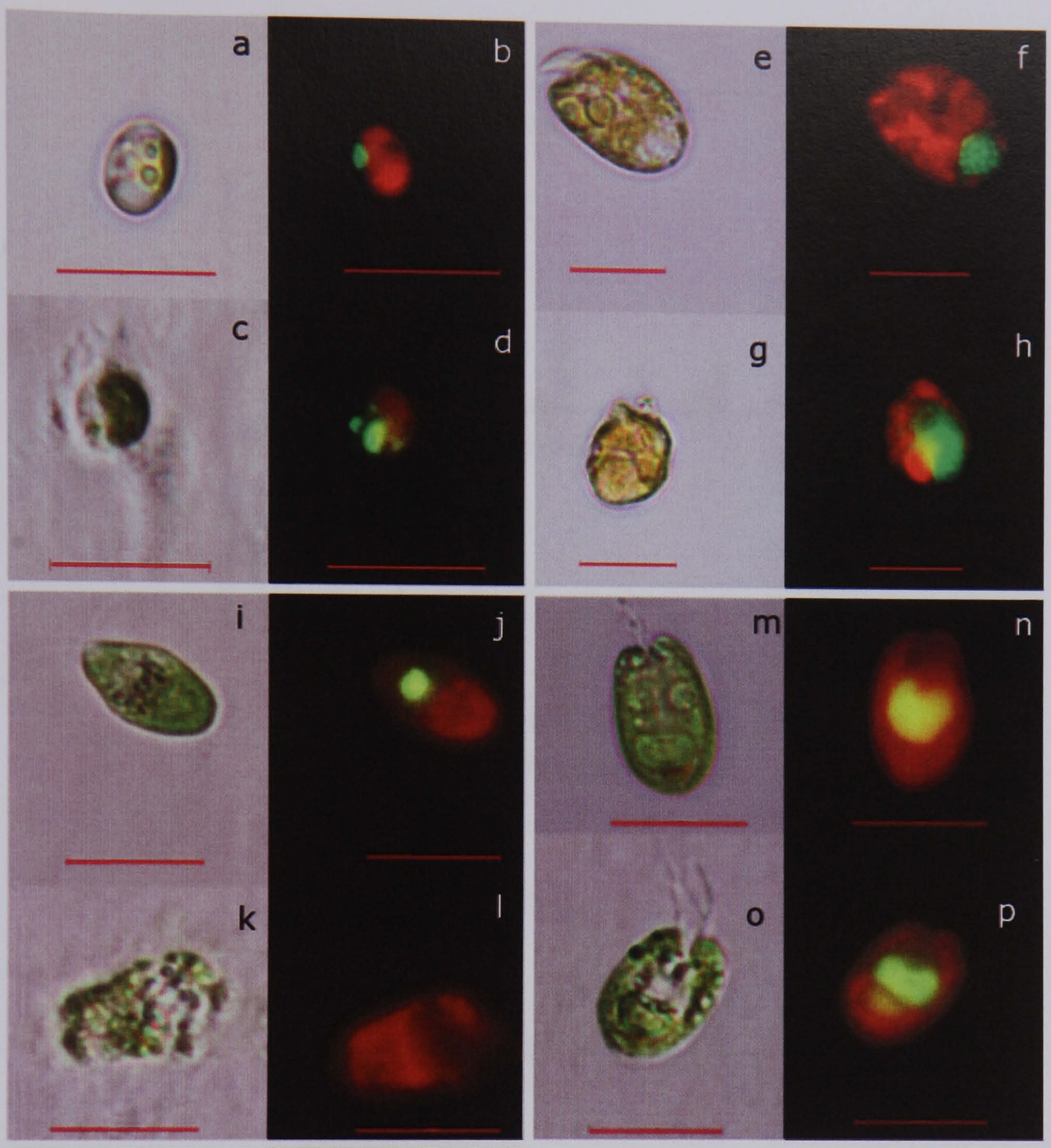


Figure III-3. Micrographs of *Isochrysis galbana* (a-d), *Amphidinium carterae* (e-h), *Dunaliella tertiolecta* (i-l), *Tetraselmis suecica* (m-p) and *Skeletonema marinoi* (q-t). First two panels of each series are transmitted and epifluorescence pictures, respectively, of untreated cells fixed with paraformaldehyde 1% and stained with the DNA stain SYBR Green I (green fluorescence from the nucleus). The last two panels of each series are transmitted and epifluorescence pictures, respectively, of cells exposed to PUA for 48 h, fixed with paraformaldehyde 1% and stained with the DNA stain SYBR Green. Note the irregular shape of nuclei in treated cells and the presence of vesicles and refringent bodies. Scale bar is 10 µm.

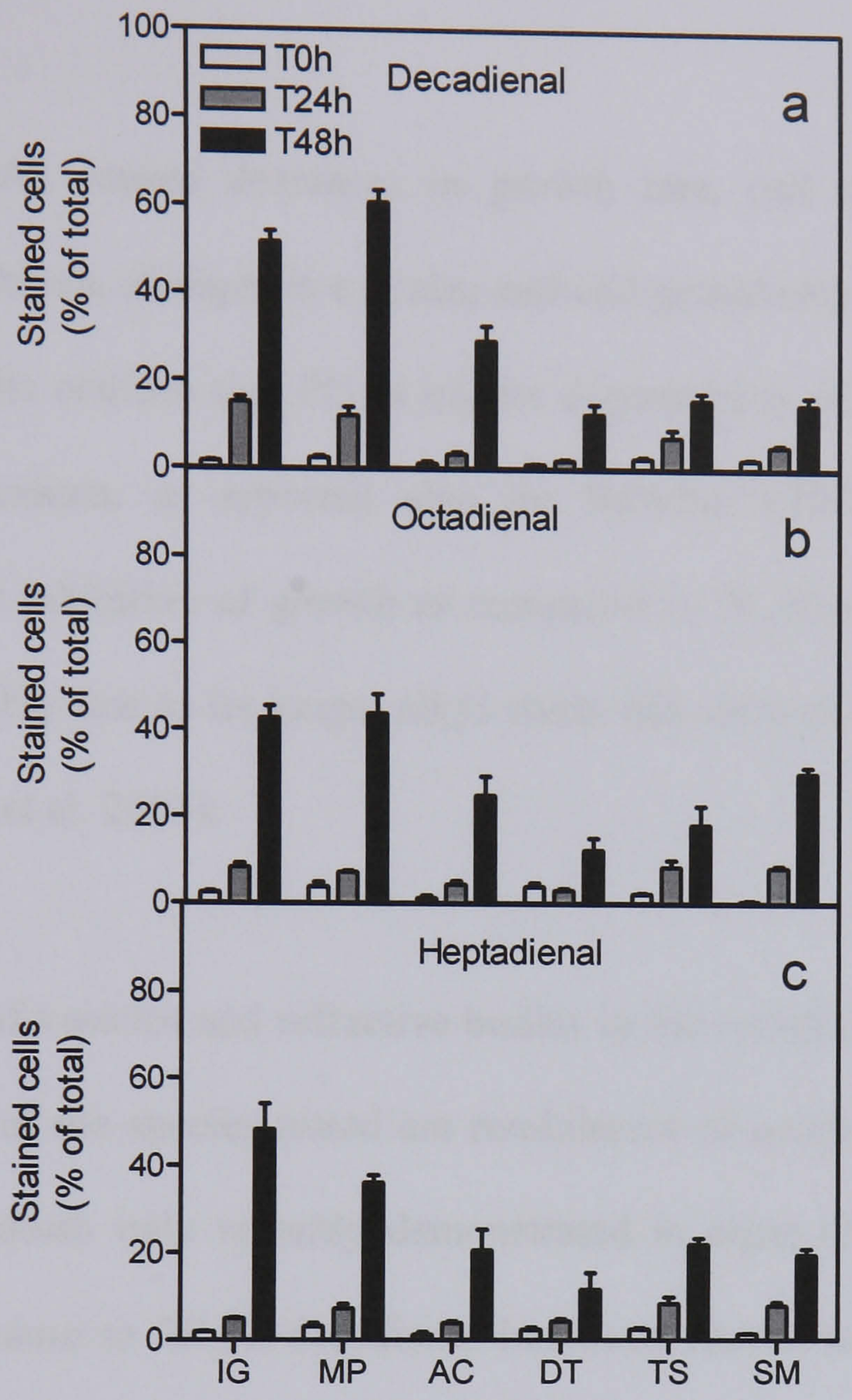


Figure III-4. Effect of the three PUAs on cell membrane permeability of *Isochrysis galbana* (IG), *Micromonas pusilla* (MP), *Amphidinium carterae* (AC), *Dunaliella tertiolecta* (DT), *Tetraselmis suecica* (TS) and *Skeletonema marinoi* (SM). Proportions of cells stained positively by SYTOX-Green (non-viable) at time 0 and after 24 h and 48 h of incubation with a) 2E,4E-decadienal, b) 2E,4E-octadienal, c) 2E,4E-heptadienal, at twice the concentration of the individual EC_{50}. Data are means of replicates with standard deviations (n = 3)

I.4. Discussion

The three PUAs caused decreases in growth rate, cell membrane disruption, chlorophyll degradation, changes in cell size and cell granulosity in the six microalgae tested. These results confirm that PUAs trigger degeneration of a broad range of key physiological processes, as reported also by Spiteller (2001). 2E,4E-decadienal induced a stronger inhibition of growth as compared to 2E,4E-octadienal and 2E,4E-heptadienal, probably due to its longer alkyl chain that increases the reactivity of the molecule (Adolph et al. 2003).

The presence of vesicles and refractive bodies in the cytoplasm and the chromatin dispersal in some of the species tested are reminiscent of apoptosis, a mechanism of programmed cell death only recently demonstrated in algae (Franklin et al. 2006). Interestingly, exposure to 2E,4E-decadienal has been shown to induce programmed cell death in many different marine organisms, including diatoms (Casotti et al. 2005), copepods and sea urchin embryos (Romano et al. 2003), suggesting a common mode of action in both animals and plants. The range of concentrations of 2E,4E-decadienal (the most widely PUA used in toxicological studies so far) affecting animals varies widely, from 0.15 to 25.92 μmol L^{-1} for crustaceans, and from 1.18 to 15.64 μmol L^{-1} for echinoderms (Caldwell et al. 2003and references therein). These concentrations appear to be higher than those identified for species in the present study, suggesting that algae may be more sensitive than animals. However, in the case of animals,

sensitive stages such as eggs are usually used and exposure times are often shorter, making it difficult to compare.

Among species, in general, our data show a higher sensitivity to PUAs of smaller phytoplankton like *I. galbana* when compared to larger species like *T. suecica* and *S. marinoi*. This could be related to the smaller size of *I. galbana* (as well as *M. pusilla*), as the higher surface to volume quotient may underlie a higher potential for uptake of lipophilic compounds such as PUAs. Differing membrane characteristics may also play a role. The more sensitive species *I. galbana* and *M. pusilla* have no distinct or mineralized cell walls (Throndsen 1997; Zhu et al. 1997) and so may be more vulnerable compared to other taxa with different cell wall properties (e.g. the silicified diatom cell wall or the highly structured cell wall of dinoflagellates). Prymnesiophytes (like *I. galbana*) are reported to have a higher total lipid content as compared to prasinophytes, chlorophytes, dinophytes and diatoms (Reitan et al. 1994) and this may also be a factor to consider to explain its higher sensitivity as compared to the other species tested. It is, in fact, reported that toxicity of lipophilic compounds is related to the total lipid content of target cells, which increases their ability to penetrate the cell membrane and to affect critical lipoprotein complexes (Hutchinson et al. 1980). Detoxification mechanisms or presence of specific target sites for PUAs may vary among taxa, and altogether, the differential sensitivity of the tested species may result in differences in their ability to compete with other species in natural waters where diatoms or other PUA-producing algae are found.

Even within diatoms (putative producers of PUAs), there are species-specific differences in sensitivity to the same PUA. *Phaeodactylum tricornutum* shows an EC_{50} for growth of 7.1 µmol L^{-1} for 2E,4E-decadienal (Vardi et al. 2006), which is much higher than what found for *Thalassosira weissflogii* (1.9 µmol L^{-1}, Casotti et al. 2005) and *S. marinoi* (2.4 µmol L^{-1}, this study). Also in this case, differences may be due to size and/or membrane mechanical or biochemical properties. But it should also be noted that none of these species are reported to produce 2E,4E-decadienal (Wichard et al. 2005a), and *P. tricornutum* does not produce PUAs at all, but other oxylipins (Pohnert et al. 2002). In this study, *S. marinoi* was particularly tolerant to 2E,4E-octadienal and 2E,4E-heptadienal, which are the main PUAs produced by this species, (d'Ippolito et al. 2003; Wichard et al. 2005a), whereas it was more sensitive to 2E,4E-decadienal. In addition, a modest stimulation of the growth at sublethal concentration of 2E,4E-octadienal and 2E,4E-heptadienal was observed but not with 2E,4E-decadienal. This suggests that *S. marinoi* has developed a moderate resistance against the compounds that it is most likely to encounter. This may also explain the Ca^{2+}-nitric oxide-mediated signalling system elicited by sublethal concentrations of 2E,4E-decadienal in *P. tricornutum* (Vardi et al. 2006), which could be interpreted as a general reaction to toxic compounds.

There are several important limitations in extrapolating the data of the present study to the natural realm. Concentrations tested appear to be high when compared to concentrations of PUAs that diatom species are reported to produce, which range from 0.01 to 9.81 fmol $cell^{-1}$ (Wichard et al. 2005a). In the present study, single model

compounds were used whereas in nature different aldehydes are released from the same cell at the same time, and so it is likely that PUA effect will act additively, as predicted by Faust et al. (2001). Moreover, due to the patchy nature of phytoplankton at sea, it is reasonable to expect high local concentrations in proximity of diatom cells. Concentration of PUAs as a function of distance from the surface of the producing cell state can be estimated using a random-walk model based on simple molecular diffusion (Berg 1983). This model uses the following formula to calculate the concentration (C) of a given compound at steady state as a function of distance from the cell (r):

$$C(r) = \frac{i}{4\pi Dr} \qquad (3)$$

where i is the PUA production rate in fmol cell^{-1} s^{-1}, r the distance from the source in μm and D the diffusion constant in μm^2 s^{-1}, which for PUAs has been conservatively calculated as ~ 450 μm^2 s^{-1} based on their stereochemistry (Tucker and Nelken 1982). *Thalassiosira rotula* is reported to produce in culture 6.35 fmol of PUA cell^{-1} in stationary phase of growth (Wichard et al. 2005a) at a rate of 0.035 fmol cell^{-1} s^{-1} (Pohnert 2000). We can therefore expect to find 6.24, 0.62 and 0.06 μmol PUA L^{-1} at 1, 10 and 100 μm in the surrounding of one single cell, respectively, soon after its lysis. This figure can be much higher at sea, if we base the calculations on values measured by Wichard et al. (2005b), which report concentrations as high as 47.7 fmol of total PUAs per cell in phytoplankton samples dominated by diatoms in the English

Channel. Based on this figure and using the same kinetic curve of Pohnert (2000) for PUA production, the rate is 0.265 fmol s^{-1}, and therefore the release of PUAs from each diatom cell can be estimated as 46.9, 4.7 and 0.5 µmol PUA L^{-1} at a distance of 1, 10 and 100 µm from the cell surface. Such concentrations are well within the significant range for affecting growth and performance of surrounding organisms. A similar approach has been used by Jackson (1980) to exclude that growth of phytoplankton in oligotrophic areas of the oceans could be supported by pulses of nutrients excreted from animals swimming near a phytoplankter, because of rapid dissipation. However, the *D* value we used is expected to be much lower than Jackson's, because of the high lipophilic nature of PUAs. Many studies have, indeed reconsidered the estimates of the *D* parameter, showing that it can be much lower (Ploug and Passow 2007). Unlike the situation in static cultures, PUA production is a continuous process, which is reinitiated when the product is removed from the enzyme (Fontana et al. 2007a), and this may contribute to maintain constant high local concentrations at sea. In addition to this, our experiments probably underestimate the potential effects of PUAs because we used nutrient-replete cultures growing logarithmically. In the previous chapter, it has been shown that that PUA production increases with age and nutrient stress of cultures, suggesting that PUAs measured in cultures are potentially underestimating true production under natural conditions. Consequently, it is reasonable to expect that PUA concentrations at sea may affect the growth of algae surrounding PUA-producing cells, even more so during blooms, when cell concentrations are higher and diatoms accumulate in patches.

Ultimately, to resolve such questions, *in situ* measurements of aldehydes are needed. Attempts to develop a sensitive method for the detection and quantification of dissolved PUAs in seawater are in progress (G. Pohnert, pers. comm.) and this is expected to represent a key step to understand the ecological role of these compounds in marine ecosystems and to stimulate new ideas for future research.

II. Differential effect of three polyunsaturated aldehydes on the growth of thirty three marine bacterial strains

(This study has been conducted within the framework of the RMP MARPLAN, and as collaboration between the Stazione Zoologica of Naples, IT, and the Observatoire Océanologique de Banyuls, FR, with Pr. Philippe Lebaron and Laurent Intertaglia)

II.1. Introduction

The diatoms *Skeletonema costatum*, *Chaetoceros socialis* and *Phaeodactylum tricornutum* are well-know for their antibacterial properties against a broad spectrum of aquacultural pathogens (Naviner et al. 1999). It has been also observed that *S. costatum* can have both an inhibitory and stimulatory effect on marine bacteria, suggesting the presence of active molecules (Bell and Mitchell 1972; Bell et al. 1974; Kogure et al. 1979). Although most of the studies focused on pathogens, the question about whether these effects related to the production of allelochemicals may be observed on co-occurring marine bacteria is of great interest. Several diatom species produce a wide range of secondary metabolites, including volatile polyunsaturated aldehydes (here abbreviated as PUAs), such as 2E,4E/Z-heptadienal, 2E,4E/Z-octadienal, and 2E,4E/Z-decadienal (Wichard et al. 2005a). PUA production in diatoms varies quantitatively and qualitatively according to the species (Wichard et al.

2005a) and to the cell physiological state (see Chap II-B). These compounds, including 2E,4E-decadienal, which has been widely used as a model aldehyde, have been shown to have a detrimental effect on cell proliferation of human cancer cell lines, egg hatching success and embryo cell division of marine invertebrates (reviewed by Ianora et al. 2006), seed germination of plants (Eom et al. 2006), and growth of fungi (Adolph et al. 2004) and phytoplankton (Casotti et al. 2005; and section I). More recently, it has been shown that 2E,4E-decadienal reduces the growth of pathogenic and non-marine bacteria (Bisignano et al. 2001; Adolph et al. 2004). Despite their importance in marine ecosystems and their presence in close vicinity to diatoms and other phytoplankton at sea, effects of these aldehydes on marine bacteria have not been tested so far.

The aim of this study is to test the effect of three diatom-derived PUAs on the growth of 33 marine bacteria belonging to different taxonomical groups, including 16 strains isolated in 2006 in the Northern Adriatic Sea during a late-winter bloom of the PUA-producing marine diatom *Skeletonema marinoi*.

II.2. Materials and Methods

II.2.1 Cultures and experimental design

Thirty three marine bacterial strains (Table III-4) were grown in marine broth medium (Marine Broth 2216, Difco Laboratories, Detroit, USA) at room temperature

in the dark. 1.5 mL of exponentially growing cultures in duplicate were exposed to the following final PUA concentrations: 13, 26, 53, 106 μmol L^{-1} of 2E,4E-decadienal; 16, 32, 65, 130 μmol L^{-1} of 2E,4E-octadienal; and 18, 36, 73, 145 μmol L^{-1} of 2E,4E-heptadienal. Initial experiments showed that lower PUA concentrations had no effect on the growth of the bacteria tested, except for *Enterococcus faecium*.

Controls were represented by cultures with no PUAs and also by cultures inoculated with ethanal and decanoic acid both at the same highest concentrations used for PUAs (150 μmol L^{-1}). These compounds had no effect on any strain tested (data not shown).

Growth was monitored by measuring light absorbance at 450 nm using a spectrophotometer connected to a multiplate reader (Victor3 multilabel counter, Perkin Elmer). Initial cell concentrations were measured by flow cytometry (FACScalibur, Becton Dickinson) on fixed (formaldehyde 2%) and stained cells (SYBR Green I, 0.025% final concentration in DMSO, Molecular Probes, Leiden, The Netherlands). Yellow-green fluorescent microspheres (0.95-μm-diameter Polysciences fluorospheres, Warrington, USA) were used as internal standards.

In order to test the hypothesis that PUAs may be used as an alternative source of carbon, a culture of *Alteromonas hispanica* was grown in minimal medium (Widdel et al. 1983), alone or amended with 2E,4E-decadienal (final concentration 106 μmol L^{-1}), 2E,4E-octadienal (final concentration 130 μmol L^{-1}) or 2E,4E-heptadienal (final concentration 145 μmol L^{-1}). As a further control, a culture of the same species was grown in minimal medium amended with 1% mannitol, 0.1% pyruvate and 0.1% proline as carbon substrates.

Table III-4. List of the bacterial species used in this study, their phylogenetic association (clade), the strain denomination, the original isolation site (origin) and depth (in meter), cell wall characteristic (cell wall) and initial cell concentration at the beginning of the experiment (cell mL^{-1}). Strains from the NW Adriatic Sea have been isolated during the 2006 late winter bloom of *Skeletonema marinoi*. The word 'bacteria' is abbreviated as 'b.' in clade.

Species	Clade	Strain	Origin	Depth	Cell wall	Initial cell concentration (cell mL^{-1})
Kocuria marina	Actinob.	DR31/18	NW Mediter. Sea	3 m	Gram +	$5.8\ 10^6$
Rhodococcus erythropolis	Actinob.	AS06/59	NW Adriatic Sea	Surface	Gram +	$4.4\ 10^6$
Fulvimarina litoralis	α -proteob.	AS06/4	NW Adriatic Sea	27 m	Gram -	$3.5\ 10^7$
Erythrobacter flavus	α -proteob.	DR41/17	NW Mediter. Sea	3 m	Gram -	$5.4\ 10^7$
Erythrobacter citreus	α -proteob.	CM31/4b	NW Mediter. Sea	3 m	Gram -	$2.3\ 10^7$
Loktanella vestfoldensis	α -proteob.	AS06/15	NW Adriatic Sea	10 m	Gram -	$1.5\ 10^7$
Oceanibulbus indoliflex	α -proteob.	AS06/19	NW Adriatic Sea	10 m	Gram -	$7.2\ 10^6$
Oceanibulbus indolifex	α -proteob.	AS06/34b	NW Adriatic Sea	2 m	Gram -	$3.5\ 10^6$
Oceanibulbus indolifex	α -proteob.	AS06/45	NW Adriatic Sea	2 m	Gram -	$1.1\ 10^7$
Oceanibulbus indolifex	α -proteob.	AS06/25	NW Adriatic Sea	10 m	Gram -	$2.6\ 10^6$
Oceanibulbus indolifex	α -proteob.	AS06/36	NW Adriatic Sea	2 m	Gram -	$6.4\ 10^6$
Paracoccus alcaliphilus	α -proteob.	AS06/34c	NW Adriatic Sea	2 m	Gram -	$1.8\ 10^7$
Phaeobacter gallaeciencis	α -proteob.	3X/A02/234	NW Mediter. Sea	0-3 m	Gram -	$1.1\ 10^7$
Roseobacter litoralis	α -proteob.	DSM 6996	Japon	Seaweed	Gram -	$3.3\ 10^7$
Sulfitobacter mediterraneus	α -proteob.	AS06/35c	NW Adriatic Sea	2 m	Gram -	$3.2\ 10^5$
Sulfitobacter pontiacus	α -proteob.	CM31/15b	NW Mediter. Sea	3 m	Gram -	$9.0\ 10^7$
Lutibacterium anuloederans	α -proteob.	AS06/5	NW Adriatic Sea	27 m	Gram -	$2.7\ 10^6$
Bacillus aquimaris	Bacilli	CZ41/18b	NW Mediter. Sea	3 m	Gram +	$1.4\ 10^7$
Enterococcus faecium	Bacilli	18III/A01/073	NW Mediter. Sea	0-3 m	Gram +	$3.5\ 10^7$
Flexibacter aggregans	Flavob.	CZ32/18a	NW Mediter. Sea	3 m	Gram -	$1.3\ 10^7$
Olleya marilimosa	Flavob.	CZ31/1c	NW Mediter. Sea	3 m	Gram -	$9.2\ 10^6$
Eurybia adriatica	Flavob.	AS06/20a	NW Adriatic Sea	10 m	Gram -	$1.1\ 10^7$
Microscilla pacifica	Flavob.	CR41/25b	NW Mediter. Sea	3 m	Gram -	$1.6\ 10^7$
Muricauda aquimarina	Flavob.	CR41/5a	NW Mediter. Sea	3 m	Gram -	$6.6\ 10^7$
Pibocella ponti	Flavob.	AS06/6	NW Adriatic Sea	27 m	Gram -	$2.6\ 10^7$
Polaribacter dokdonensis	Flavob.	AS06/17	NW Adriatic Sea	10 m	Gram -	$4.4\ 10^7$
Roseivirga echinicomitans	Flavob.	Biosli/23	SW Pacific	5 m	Gram -	$3.5\ 10^7$
Alteromonas hispanica	γ -proteob.	14III/A01/003	NW Mediter. Sea	0-3 m	Gram -	$1.1\ 10^7$
Alteromonas maclodeii	γ -proteob.	CZ32/6b	NW Mediter. Sea	3 m	Gram -	$1.4\ 10^7$
Alteromonas marina	γ -proteob.	CM31/5a	NW Mediter. Sea	3 m	Gram -	$6.7\ 10^6$
Alteromonas stellipolaris	γ -proteob.	CR31/20a	NW Mediter. Sea	3 m	Gram -	$6.8\ 10^6$
Pseudomonas oryzihabitans	γ -proteob.	AS06/53a	NW Adriatic Sea	Surface	Gram -	$1.7\ 10^5$
Vibrio splendidus	γ -proteob.	AS06/30	NW Adriatic Sea	2 m	Gram -	$1.9\ 10^7$

II.2.2 Preparation of chemicals

2E,4E-heptadienal, 2E,4E-octadienal, 2E,4E-decadienal, ethanal and decanoic acid were obtained commercially from Sigma-Aldrich Inc. (Milano, Italy). Working solutions were prepared as described in section I. Toxicity of the methanol solvent was tested for all bacterial strains and was above 7 µL of pure methanol per ml of culture (data not shown); therefore the amount of aldehyde solution in each test was kept always below this threshold.

II.2.3 Statistical Analyses

Normal distribution and equal variance of the data were tested and a Dunnett's Multiple Comparison post-test (Prism 4, GraphPad Software, San Diego, USA) was used to assess the PUA concentrations inducing a significant effect when compared to the controls. The Lowest Observed Effect Concentration (LOEC) was estimated as the lowest PUA concentration inducing an effect different than the control with 95% significance.

Because the number of concentrations tested did not provide enough data to calculate the statistical no effect concentration or the EC_{50} (as used in the section I), LOEC was used to estimate the effect of the PUAs on bacteria.

II.3. Results

A group of 12 strains showed no difference with the control when inoculated with PUA concentrations up to 106, 130, and 145 µmol L^{-1} of 2E,4E-decadienal, 2E,4E-octadienal and 2E,4E-heptadienal, respectively (Table III-5). These included 3 out of 6 γ-Proteobacteria, 5 out of 15 α-Proteobacteria and 4 out of 8 Flavobacteria. 2E,4E-heptadienal had no effect on Flavobacteria except for *Muricauda aquimarina*, while *Microscilla pacifica* and *Pibocella ponti* showed growth inhibition only at the highest concentrations of 2E,4E-decadienal and 2E,4E-octadienal (Table III-5). The response of the flavobacteria *Roseivirga echinicomitan*s and the γ-proteobacteria *Alteromonas marina* to PUAs exposure is given as an example (Figure III-5). Most of the strains isolated during a bloom of the PUA-producing diatom *Skeletonema marinoi* (see Table III-4) showed no effect upon PUA inoculation, only *Pseudomonas oryzihabitans* and *Paracoccus alcaliphilus* were inhibited at PUA concentrations below 106, 130, and 145 µmol L^{-1} for 2E,4E-decadienal, 2E,4E-octadienal and 2E,4E-heptadienal, respectively (Table III-5).

Table III-5. List of the bacterial species used in this study, their phylogenetic affiliation, and values of the Lowest Observed Effect Concentration (LOEC) of 2E,4E-decadienal, 2E,4E-octadienal and 2E,4E-heptadienal (in μmol L^{-1}). LOEC is the lowest concentration tested that induced a significant response on cell density relative to the control, calculated by using Bonferroni post-test (p <0.05). No effect means that the highest aldehyde concentrations tested (106, 130 and 145 μmol L^{-1} of 2E,4E-decadienal, 2E,4E-octadienal and 2E,4E-heptadienal, respectively) did not induce any effect different from the control (p >0.05). # indicates aldehyde concentrations which induced an increase of the cell growth in the cultures.

Phylogenetic affiliation		Lowest Observed Effect Concentration		
Species	Clade	2E,4E-decadienal	2E,4E-octadienal	2E,4E-heptadienal
Kocuria marina	Actinobacteria	13	16	18
Rhodococcus erythropolis	Actinobacteria	106	no effect	no effect
Fulvimarina litoralis	α -proteobacteria	no effect	no effect	no effect
Erythrobacter flavus	α -proteobacteria	106	no effect	no effect
Erythrobacter citreus	α -proteobacteria	106	130	145
Loktanella vestfoldensis	α -proteobacteria	106	130	145
Oceanibulbus indoliflex	α -proteobacteria	no effect	130	no effect
Oceanibulbus indolifex	α -proteobacteria	no effect	130	no effect
Oceanibulbus indolifex	α -proteobacteria	no effect	no effect	no effect
Oceanibulbus indolifex	α -proteobacteria	106	130	145
Oceanibulbus indolifex	α -proteobacteria	106	130	145
Paracoccus alcaliphilus	α -proteobacteria	106	65	73
Phaeobacter gallaeciencis	α -proteobacteria	13	16	18
Roseobacter litoralis	α -proteobacteria	13	16	18
Sulfitobacter mediterraneus	α -proteobacteria	no effect	no effect	no effect
Sulfitobacter pontiacus	α -proteobacteria	no effect	no effect	no effect
Lutibacterium anuloederans	α -proteobacteria	no effect	no effect	no effect
Bacillus aquimaris	Bacilli	53	65	73
Enterococcus faecium	Bacilli	3	4	4
Flexibacter aggregans	Flavobacteria	no effect	no effect	no effect
Olleya marilimosa	Flavobacteria	no effect	no effect	no effect
Eurybia adriatica	Flavobacteria	13 #	16 #	18 #
Microscilla pacifica	Flavobacteria	106	130	no effect
Muricauda aquimarina	Flavobacteria	53	65	73
Pibocella ponti	Flavobacteria	no effect	130	no effect
Polaribacter dokdonensis	Flavobacteria	no effect	no effect	no effect
Roseivirga echinicomitans	Flavobacteria	no effect	no effect	no effect
Alteromonas hispanica	γ -proteobacteria	13 #	16 #	18 #
Alteromonas maclodeii	γ -proteobacteria	53	65	73
Alteromonas marina	γ -proteobacteria	no effect	no effect	no effect
Alteromonas stellipolaris	γ -proteobacteria	no effect	no effect	no effect
Pseudomonas oryzihabitans	γ -proteobacteria	26	16	18
Vibrio splendidus	γ -proteobacteria	no effect	no effect	no effect

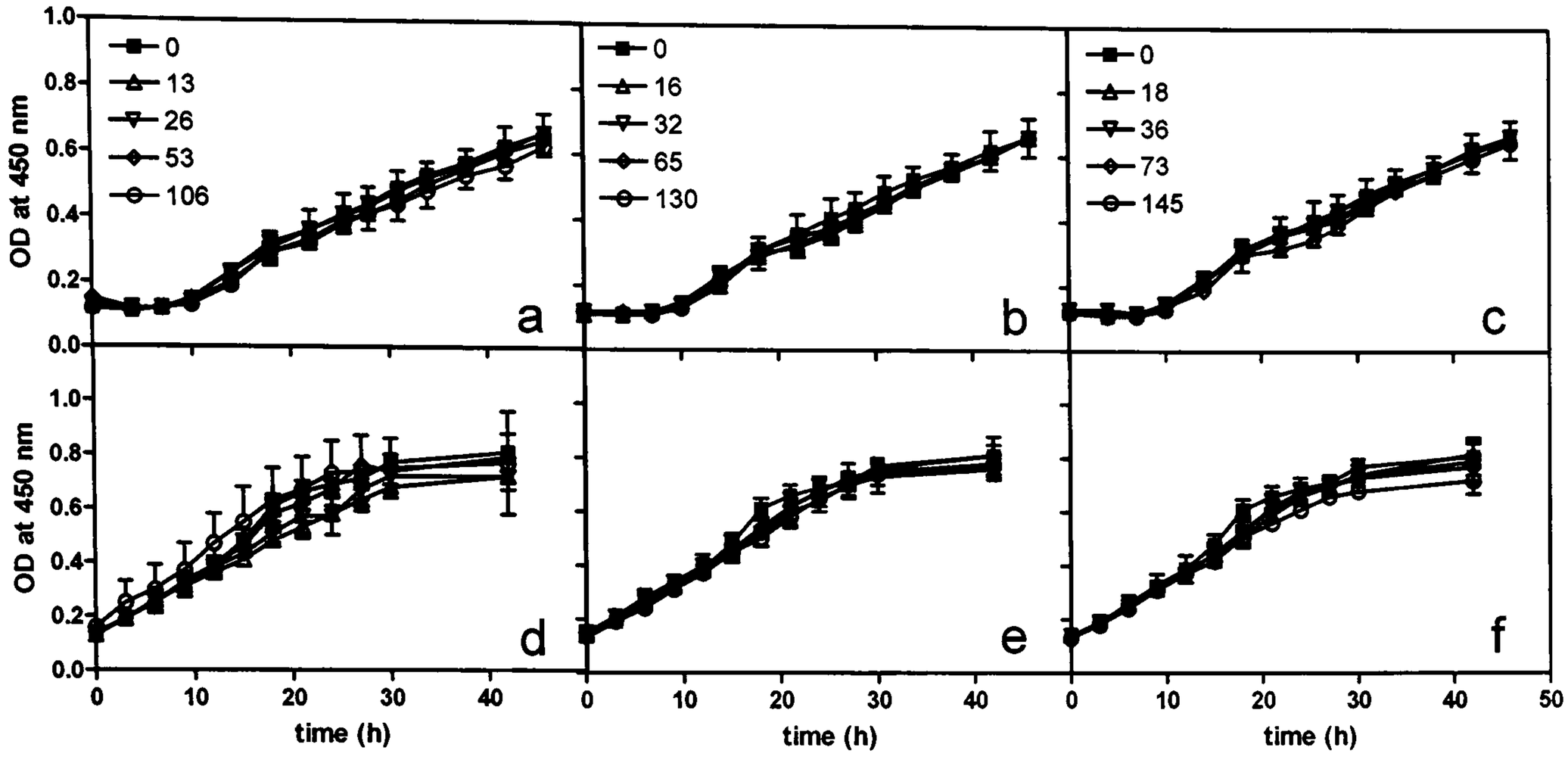


Figure III-5. Typical growth curves of the bacterial strains showing no effect of aldehydes when compared to the controls. O. D. is Optical Density measured at 450 nm, which is an estimate of cell density. The plots show the results from *Roseivirga echinicomitans* (a, b and c, respectively) and *Alteromonas marina* (d, e and f, respectively) when inoculated with the concentrations indicated on each plot of 2E,4E-decadienal (a and d), 2E,4E-octadienal (b and e), and 2E,4E-heptadienal (c and f) (μmol L^{-1}). Data are means of independent replicates with standard deviation (n = 2).

A concentration-dependent growth reduction was observed for 16 strains exposed to 2E,4E-decadienal, 17 strains exposed to 2E,4E-octadienal and 13 strains exposed to 2E,4E-heptadienal (Table III-5). These included 2 Bacilli, 2 Actinobacteria, 10 out of 15 α-proteobacteria, 2 out of 6 γ-proteobacteria and 3 out of 8 Flavobacteria. *Enterococcus faecieum* was the most sensitive to PUAs with the lowest observed effect concentration (LOEC) of 3 μmol L^{-1} for 2E,4E-decadienal, 4 μmol L^{-1} for 2E,4E-octadienal and 4 μmol L^{-1} for 2E,4E-heptadienal. The response of the flavobacteria *Muricauda fluvescens* and the α-proteobacteria *Roseobacter litoralis* to PUA exposure is given as an example (Figure III-6). *Kocumaria marina*, *Roseobacter litoralis* and *Phaeobacter gallaeciencis* were particularly sensitive to PUAs with growth inhibition occurring at 13, 16 and 18 μmol L^{-1} for the three PUAs, respectively. A wide range of LOEC values was observed within each bacterial clade and family. As an example, within the Bacilli, *Bacillus aquimaris* showed growth inhibition at concentrations 16 times higher than *E. faecieum*, and within the Rhodobacteraceae, *Loktanella vestfoldensis* showed LOEC values 8 times higher than *Roseobacter litoralis* and *Phaeobacter gallaeciencis* (Table III-5). A wide range of sensitivity to PUAs was also observed within the same genus (*Alteromonas*) or even among different strains belonging to the same species (*Oceanibulbus indolifex)* (Table III-5).

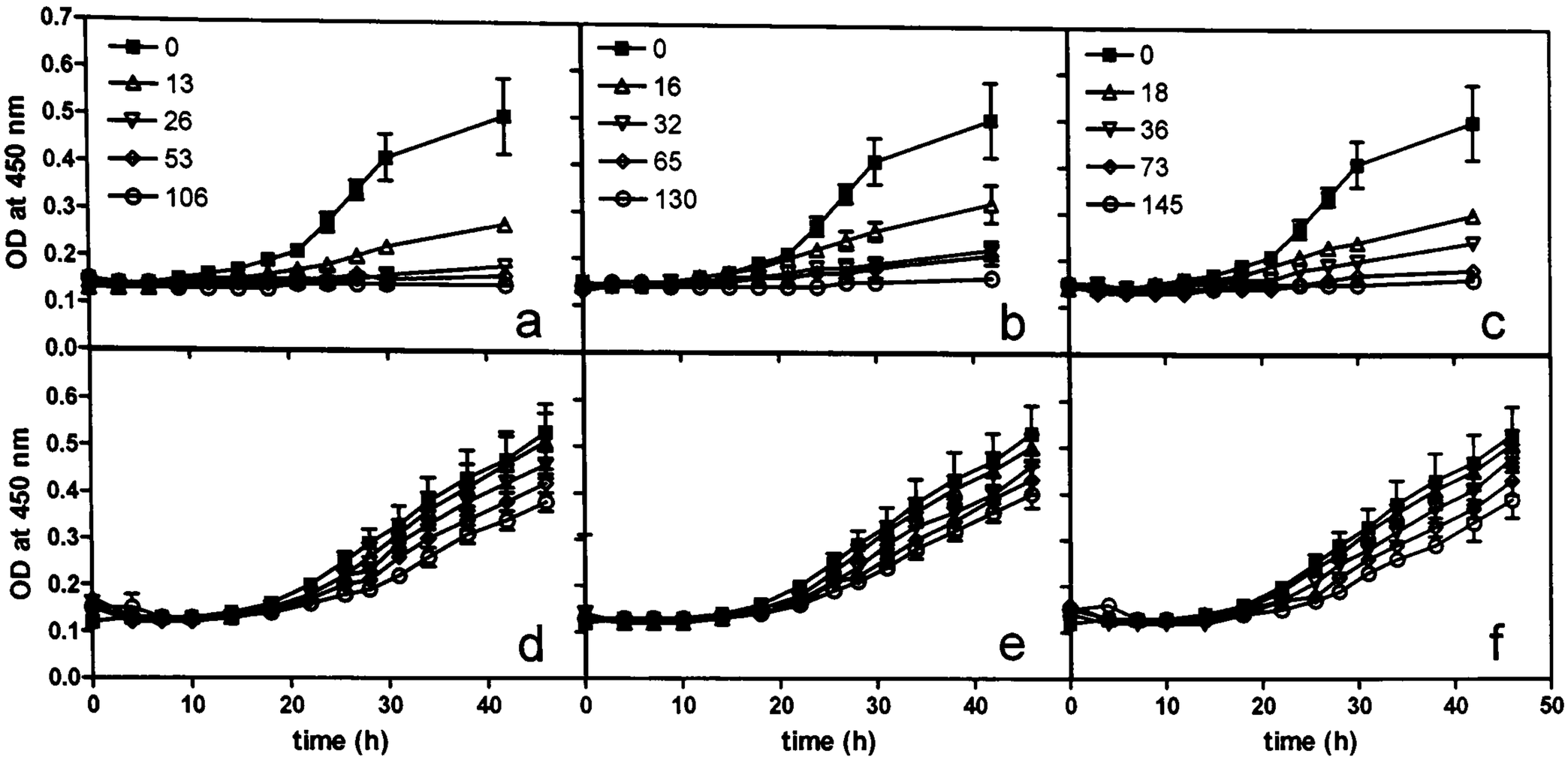


Figure III-6. Typical growth curves of the bacterial strains showing a negative effect (reduction) of aldehydes on cell growth. O. D. is Optical Density measured at 450 nm, which is an estimate of cell density. The plots show the results from *Muricauda fluvescens* (a, b and c, respectively) and *Roseobacter litoralis* (d, e and f, respectively) when inoculated with the concentrations indicated on each plot of 2E,4E-decadienal (a and d), 2E,4E-octadienal (b and e), and 2E,4E-heptadienal (c and f) (μmol L^{-1}). Data are means of independent replicates with standard deviation (n = 2).

A significant increase of the maximum cell concentration was observed for *Alteromonas hispanica* and *Eurybia adriatica* exposed to PUAs (Figure III-7). For both species, LOEC values were13, 16 and 18 µmol L^{-1} of 2E,4E-decadienal, 2E,4E-octadienal and 2E,4E-heptadienal, respectively. When inoculated in minimal medium with or without PUAs *A. hispanica* was not able to grow except when it was provided with carbon in the form of mannitol, pyruvate or proline (Figure III-8), suggesting that PUAs were not used as an external source of carbon but rather acted as growth co-factors.

II.4. Discussion

Our data show a differential sensitivity of marine bacteria to polyunsaturated aldehydes such as those produced by marine diatoms and other phytoplankton. The specificity of action of PUAs is due to the two double bonds in the carbonyl chain that influence the polarity of the aldehydic group. This is confirmed by the lack of response of bacteria to ethanal, which has a saturated aldehyde structure, and also to decanoic acid, which has similar carbon chain but no aldehydic group, confirming previous results with other monounsaturated or saturated aldehydes (Bisignano et al. 2001; Adolph et al. 2004; Casotti et al. 2005).

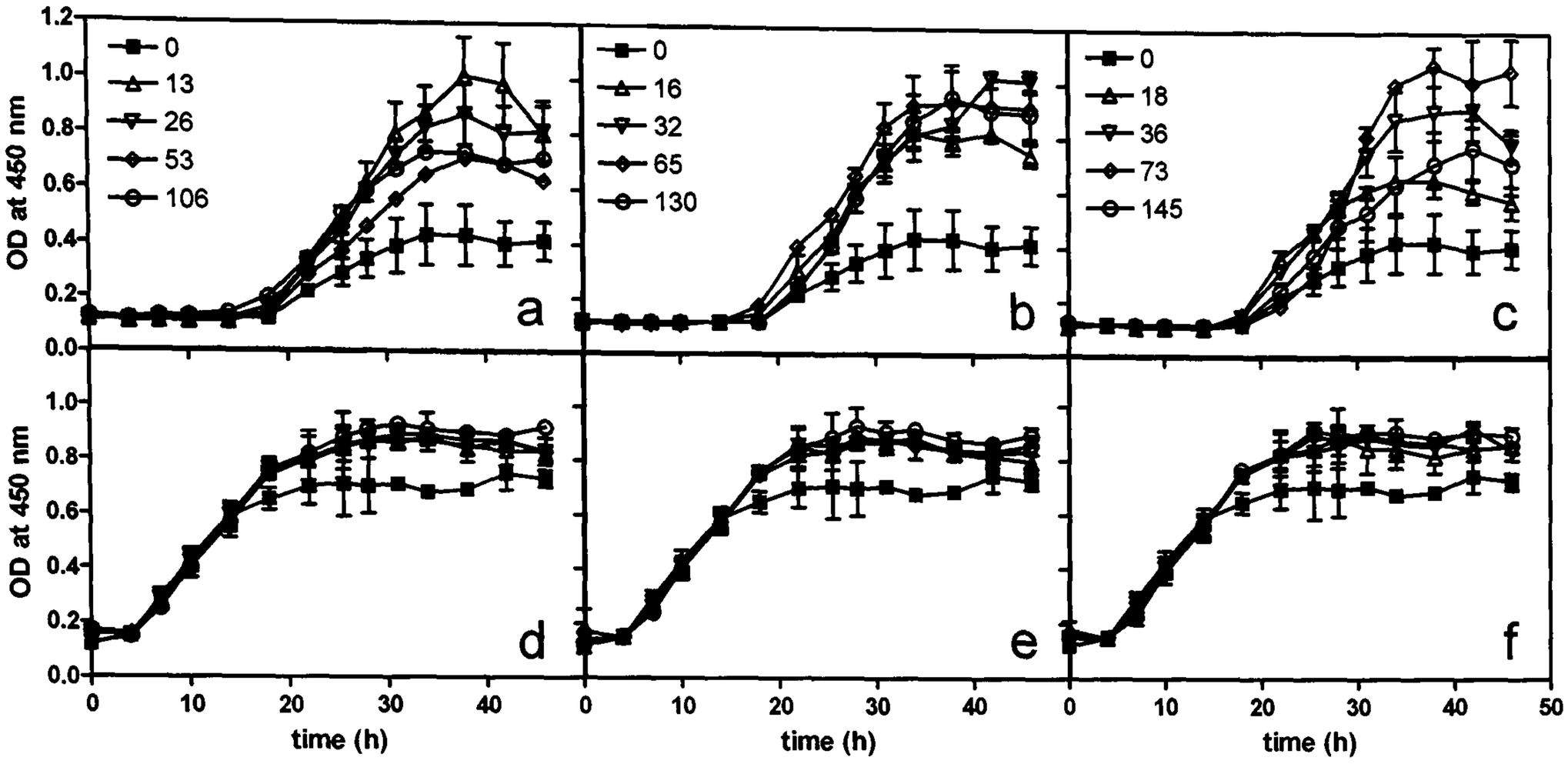


Figure III-7. Growth curves of *Eurybia adriatica*. (a, b and c) and *Alteromonas hispanica* (d, e and f) showing the stimulating effect (increase) of aldehydes on cell growth. O. D. is Optical Density measured at 450 nm, which is an estimate of cell density. Aldehyde concentrations are indicated on each plot for 2E,4E-decadienal (a and d), 2E,4E-octadienal (b and e), and 2E,4E-heptadienal (c and f) (μmol L^{-1}). Data are means of independent replicates with standard deviation (n = 2).

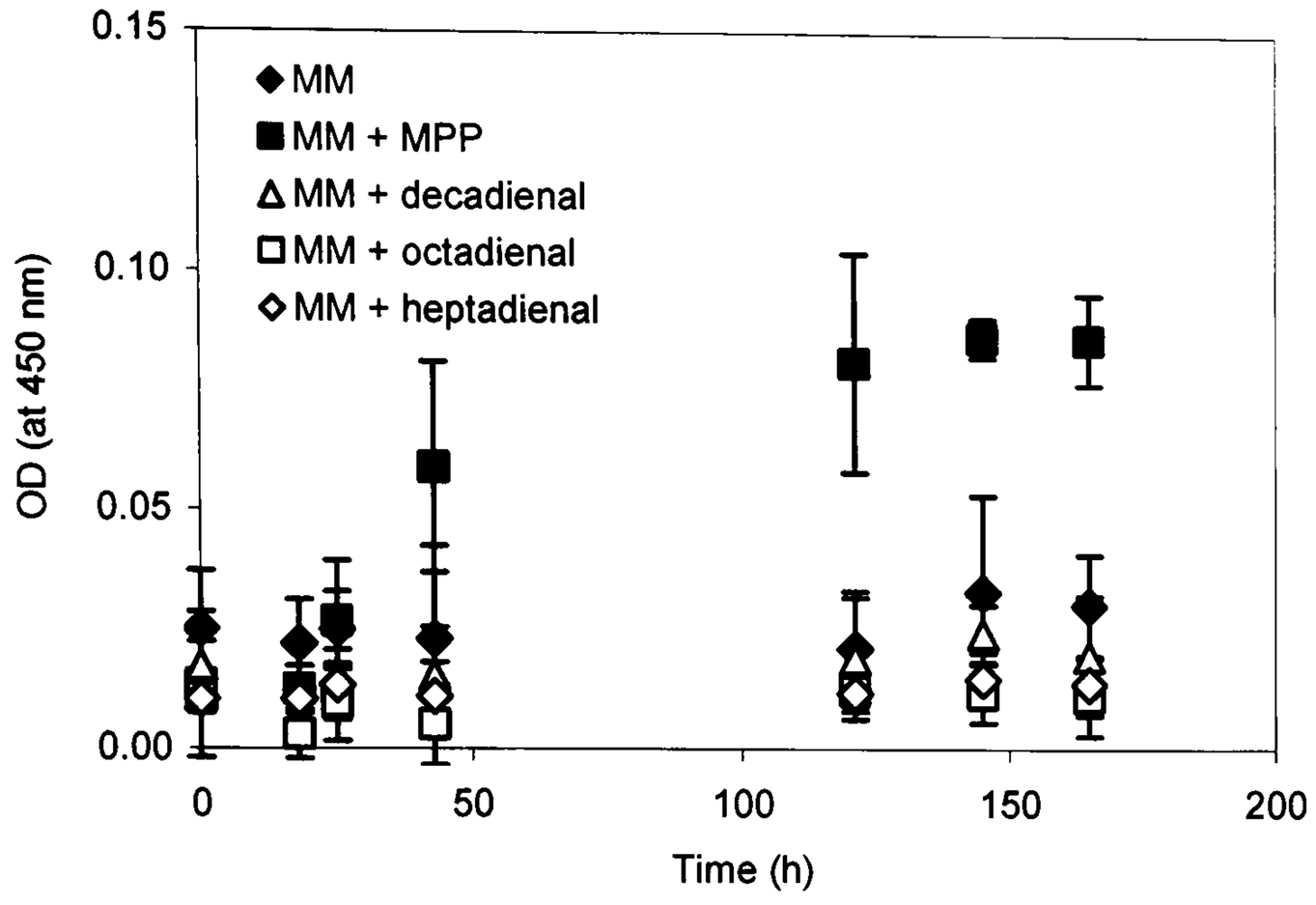


Figure III-8. Growth curves of *Alteromonas hispanica* grown in minimal medium (MM), MM amended with 1% Mannitol, 0.1% Pyruvate, and 0.1% Proline (MPP), MM amended either with 106 μmol L^{-1} 2E,4E-decadienal (MM + decadienal), or with 130 μmol L^{-1} 2E,4E-octadienal (MM + octadienal), or with 145 μmol L^{-1} 2E,4E-heptadienal (MM + heptadienal). O. D. is Optical Density measured at 450 nm, which is an estimate of cell density. Data are means of independent triplicates with standard deviation (n = 3).

The effective PUA concentrations reported in this study are consistent with those observed on pathogenic bacteria, whose effective concentrations ranged from 12.5 up to 882 μmol L^{-1} (Bisignano et al. 2001), but they are mostly one to two orders of magnitude higher than those needed to induce an effect in phytoplankton cells (Casotti et al. 2005; and section I). This suggests that bacteria are more resistant to PUAs than algae. The toxicity of PUAs has been attributed to their ability of interfering lipoprotein complexes in membranes, which should be exerted mainly at the cell surface (Hutchinson et al. 1980). In phytoplankton, it has been suggested that different responses to PUA are to be related, among other factors, to cell wall properties (see section I). Consequently, the remarkable resistance of bacteria shown in this study may also be attributed to their lipid bilayer cell wall, providing a barrier of generally low permeability, and to the additional Gram- negative outer membrane, which has been shown to represent a very effective protection against lipophilic compounds (Nikaido 1994). Most plant secondary metabolites are less active against Gram-negative than against Gram-positive bacteria (Cowan 1999), the latter being more permeant to small molecules (Scherrer and Gerhardt 1971). In our study, the Gram-positive *Enterococcus faecium* was the most sensitive species among those tested, with LOEC values in the same range of those of phytoplankton. However, another Gram-positive, *Rhodococcus erythropolaris* was also very resistant, indicating that some other factor(s) come(s) into play. This may be represented by detoxification mechanisms by means of degradation or inactivation of PUAs by specific enzymes, such as the enzyme NADPH dehydrogenase (also called "old yellow enzyme") which is known to be involved in the degradation of unsaturated carbonyl compounds

(Trotter et al. 2006). In addition, the differential responses to PUA exposure observed among the α-proteobacteria *Oceanibulbus indolifex* strains may indicate that the sensitivity to these compounds is dependent on various factors, such as the ability to produce quorum sensing signalling molecules and other secondary metabolites, as observed among different strains of *O. indolifex* (Wagner-Dobler et al. 2004, 2005).

Phytoplankton and bacteria live in tight association in aquatic environments, controlling the dynamics and succession of each others (Cole 1982; Doucette 1995; Sapp et al. 2007). Several researchers have reported evidence for species-species interactions between bacteria and phytoplankton, which has led to the conclusion that bacteria play a major role in controlling phytoplankton dynamics (Imai et al. 1993; Yoshinaga et al. 1997). There is also evidence that organic matter produced by different phytoplankton groups differentially affect the bacterial communities. Free-living bacteria, indeed, are usually phylogenetically distinct from those living attached to phytoplankton in the sea (Delong et al. 1993) and in diatom cultures (Grossart et al. 2005), which indicates that selective forces must be involved in the control of the bacterial community composition.

Bacterial succession during algal blooms has been related to the physiological status of phytoplankton cells, which control the organic matter composition and/or produce antimicrobial compounds (Jasti et al. 2005). Although there are only few reports focusing on the interactions between diatoms and bacteria, our data support the hypothesis that PUAs play a role in driving the bacterial community diversity, with

neutral, positive or negative interactions depending on the bacterial species. *Alteromomas* spp., for example, showed a sharp resistance or stimulation to PUAs. Indeed, different strains of this genus have been isolated from diatom cultures (Sapp et al. 2007), and Stewart et al. (1997) showed that the interaction between a diatom and the bacterium can result beneficial to both. We also obtained clear evidence that PUAs may be a source of carbon for some species and be toxic for other species. The *Sulfitobacter* species tested in this study were resistant to PUAs and were isolated from both the western Mediterranean Sea and the Adriatic Sea. This is congruent with the fact that species of this genus are commonly found in association with phytoplankton blooms (Rooney-Varga et al. 2005; Grossart et al. 2005). *Sulfitobacter* species were isolated from diatoms by Sapp et al. (2007) and this genus was found in the fraction of bacteria attached to the toxic dinoflagellate *Alexandrium fundyense* (Rooney-Varga et al. 2005). The strain identified as similar to *Roseobacter litoralis* ($DSM6996^T$) was, instead, sensitive to low concentrations of PUAs. This may explain why Grossart et al (2005) found that this genus was more related to the free-living fraction of bacteria in diatom cultures, than to the attached fraction which was instead, dominated by representatives of the *Flavobacteria-Sphingobacteria* group. Some Flavobacteria tested in our study were also able to grow in the presence of PUAs at low concentrations whereas some others were not affected by PUAs.

Whether PUA concentrations used in this study are relevant for natural samples is hard to know, since the different PUAs released at the same time by the cell may act additively and also environmental factors may modulate their effect. A synergetic

antibacterial effect when different aldehydes are employed in combination has also been observed by Bisignano et al. (2001). However, it is possible to estimate local concentrations around a dying diatom cell based on a simple diffusion model (Berg 1983). The gradient of PUA concentration is calculated according to equation 3 described in section 1. The resulting "pulse" of PUAs liberated by one diatom cell can be estimated as 468.6, 46.9 and 4.7 μmol L^{-1} at 0.1, 1 and 10 μm from the cell surface, respectively. These are in the range of concentrations eliciting a response on bacteria in this study, at least on some of the strains tested. It should also be considered that PUA production is a continuous process which can be reinitiated when the product is diffused away from the enzyme (Fontana et al. 2007a) and this may contribute to maintain constant high local concentrations in proximity of diatom cells. Although a decrease of PUAs can result at sea from adsorption, photolysis, chemical transformation, uptake and breakdown by other microorganisms, it is very likely that concentrations of PUAs released in the seawater by a dying diatom cell are high enough to influence both free-living and attached bacteria. During diatom blooms, when cell concentrations are high and diatoms accumulate in patches, bacterial groups that are resistant or are able to detoxify PUAs will have an advantage and should be able to outcompete other less resistant bacterial groups.

There are intrinsic artefacts that must be taken into account in the extrapolation of the present results to the natural realm, e.g. the effect of PUAs has been investigated only on cultivable bacteria which probably do not fully represent natural bacterial communities and populations. Experiments on natural communities inoculated with

PUAs are in progress (Drs. Cecilia Balestra, Stazione Zoologica di Napoli) and may help to better understand the ecological role of these compounds in marine ecosystems.

III. Exposure to sublethal concentrations of polyunsaturated aldehydes leads to resistance in diatoms

(Results presented here represent a partial contribution to the published paper by Vardi, A., Formiggini, F., Casotti, R., De Martino, A., Ribalet, F., Miralto, A., and Bowler, C., 2006. A stress surveillance system based on calcium and nitric oxide in marine diatoms. PLoS Biology 4, 411-419).

III.1. Introduction

Previous studies have reported the acute toxicity of the short-chain polyunsaturated aldehydes (PUAs), such as 2,4-decadienal, on copepods and other various invertebrates (Miralto et al. 1999; Caldwell et al. 2003; Ianora et al. 2004). Furthermore, it has now emerged that these same aldehydes are toxic to various phytoplankton species (see section I) and can trigger a process bearing the hallmarks of programmed cell death in diatoms (Casotti et al. 2005). The production of these compounds varies among species and strains (Wichard et al. 2005a) and is strongly modulated by stress induced by abiotic factors, such as nutrient limitation, extreme irradianceand UV exposure (see Chap II-B). Therefore, it is most likely that organisms might be exposed to a range of PUA concentrations at sea.

When assessing the acute toxicity of polyunsaturated aldehydes (PUAs) on phytoplankton, the diatom PUA-producing *Skeletonema marinoi* showed resistance to self-produced PUAs as compared to other phytoplankton species, and a modest stimulation of the growth rate was observed at sublethal concentrations of these PUAs (0.8-0.9 μmol L^{-1}). The phenomenon that toxic substances may at low, sublethal concentrations have a stimulating effect on growth, is called hormesis, and is widespread among various taxonomical groups (including microalgae) and a range of toxic compounds (Stebbing 1998). For now, there is no explanation for hormesis other than a self-regulating process operating through some kind of feedback mechanism (Stebbing 1998). None of the reported studies was explicitly aimed at the detection of this phenomenon and therefore little is known about the ecological effect that sublethal concentrations of toxic compounds may have on population dynamics (Calabrese and Baldwin 2003)

The data presented in this subsection explore how diatoms perceive and respond to sub-lethal concentrations of aldehydes. Experiments were performed on the diatom *Phaeodactylum tricornutum* exposed to 2E,4E-decadienal. The strain used has been stably transformed with a reporter gene for Ca^{2+} variations, whose analysis was the main aim of the Vardi et al. (2006) paper. 2E,4E-decadienal was chosen as the model aldehyde since its reactive properties are currently being tested on various animal, plant, and unicellular systems (see Caldwell et al. 2003 and references therein).

III.2. Material and Methods

III.2.1 Algal cultures and experimental design

The diatom *Phaeodactylum tricornutum* Bohlin strain CCMP 632 was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Boothbay Harbor, USA). Semi-continuous cultures were grown axenically in seawater enriched with nutrients as in f/2 medium (Guillard 1975) in a culture room at 20 °C with a 12h -12h light-dark cycle under a photon flux density of 100 µmol m^{-2} s^{-1} provided by white fluorescent tubes (Philips TLD 36W/950). Exponentially growing cultures at cell densities from 1.10^5 to 5.10^5 cell mL^{-1} were used for all experiments.

Cells were pretreated with 660 nmol L^{-1} of 2E,4E-decadienal for 2 h prior to subsequent addition of 13.2 µmol $L^{-1}$of decadienal and compared to a single dose treatment of 13.2 µmol L^{-1} of decadienal. Cell concentration and viability were followed for 60 h. Cells were resuspended in 2,4-decadienal free medium and cell concentration was monitored for 5 days.

III.2.2 PUA preparation

2E,4E-decadienal was obtained from Sigma-Aldrich Inc. (Milano, Italy). Working solutions were prepared by diluting the stock in absolute methanol (ROMIL, Cambridge, UK) at room temperature. The effective PUA concentration of the

working solution was assessed spectrophotometrically before inoculation using a wavelength of 274 nm and a specific molar absorption coefficient of 3.1×10^4 (Pippen and Nonaka 1958). Methanol had no effect on growth up to 7 µl of 100% methanol per ml of culture (data not shown) and the amount of aldehyde solution in each test was kept well below this threshold.

III.2.3 Flow cytometry, cellular parameters and cell viability

A Becton-Dickinson FACScalibur flow cytometer equipped with an air-cooled 488 nm argon-ion laser was used to estimate cell concentrations and percentage of viable cells. Data acquisition (10^4 cells on average for each sample) and analysis were performed using CellQuest software (Becton-Dickinson, San José, USA). The percentage of viable cells was assessed using the vital stain SYTOX Green (Molecular Probes, Leiden, The Nederlands) (Casotti et al., 2005). This stain does not penetrate live cells but only those with compromised plasma membranes. Optimal final concentration used was 500 nmol L^{-1} and time of incubation was 5 min (Raffaella Casotti, pers. comm.). The green fluorescence of stained cells was collected through a 530/30-nm bandpass filter.

III.3. Results

The culture pretreated with sublethal concentration of 2,4-decadienal (660 nmol L^{-1}) for 2 h prior to subsequent addition of a higher concentration (13.2 µmol L^{-1}) displayed remarkable differences from the population that was not pretreated with 2,4-decadienal. Substantial cell death was already visible after 33 h in the non-pretreated culture, whereas cell death in the pretreated culture was significantly delayed (Figure III-9). After 60 h, cell death in the preconditioned population was around 40%, whereas more than 70% of cells in the non-preconditioned population were stained with the cell death indicator Sytox Green (Figure III-9). Furthermore, resuspension of the same cultures in decadienal-free fresh medium revealed a notable difference in growth rates, with a 6-fold increase in cell concentration in the acclimated population compared with cultures from non-acclimated cells after 5 d (Figure III-9). Interestingly the pretreated culture could fully recover, whereas the non-acclimated culture was not viable and ultimately collapsed (Figure III-9, inset).

III.4. Discussion

The results show that pretreatment with sublethal concentration of 2E,4E-decadienal induce a resistance to normally lethal concentrations.

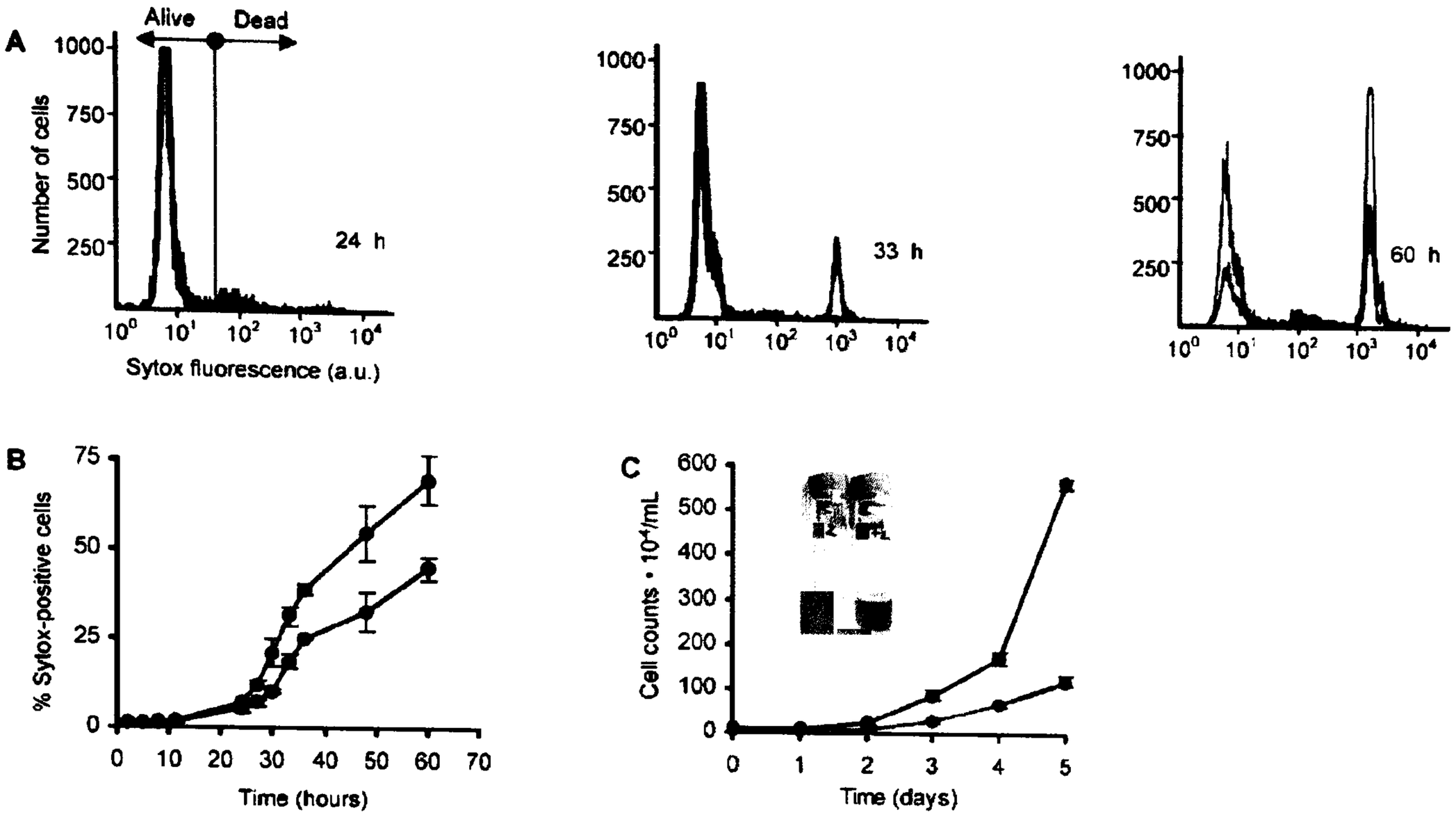


Figure III-9. Sublethal 2E,4E-decadienal concentrations can induce resistance to lethal concentrations in *P. tricornutum*. Cells were pretreated with 660 nmol L^{-1} of 2E,4E-decadienal for 2 h prior to subsequent addition of 13.2 µmol L^{-1} of decadienal (blue) and compared to a single dose treatment of 13.2 µmol L^{-1} of decadienal (green). A and B) Cell death was assayed by flow cytometry both qualitatively in cytograms (A) and quantitatively (B) using Sytox Green at the indicated time points. (C) Cell growth curves following resuspension of pretreated and non-pretreated cells in decadienal - free fresh medium 60 h after the 13.2 µmol L^{-1} decadienal treatment. The time scale indicates the days following resuspension. Inset shows photograph of the two cultures taken 2 wk after resuspension starting from an initial inoculum of 5.10^4 cells ml^{-1}. Representative data from at least five experiments are shown in (A – C). a.u., arbitrary units. (from Vardi et al., 2006)

Since aldehydes such as 2E,4E-decadienal are released by wounded diatom cells (Pohnert 2002), it is likely that this aldehyde is used as an infochemical which regulates cell fate in diatom populations at concentrations one order of magnitude lower than previously reported for effects on copepods and diatoms (Miralto et al. 1999; Ianora et al. 2004; Casotti et al. 2005).

The wider study to which these results belong (Vardi et al. 2006) examined in *P. tricornutum* the molecular mechanism responsible for these contrasting responses, i. e. induced resistance versus death. 2E,4E/Z-decadienal triggers intracellular calcium transients and the generation of nitric oxide (NO) by a calcium-dependent NO synthase-like activity, which results in cell death. The pretreatment of cells with sublethal concentrations of aldehyde stimulate calcium- and NO-based signaling systems and thereby induces resistance to successive aldehyde exposure. Perception of sublethal concentrations of aldehydes by cells in the vicinity of the producing cells could sensitize calcium- and NO-based signalling systems to induce resistance to successive aldehyde exposure. This therefore would provide an early-warning protective mechanism similar to terrestrial plants, which produce volatile organic compounds, such as jasmonic acid which provides some immunity during plant – plant – herbivore interactions (Baldwin et al. 2002).

When stress conditions increase during a bloom and trigger cell lysis with subsequent production of PUAs, aldehyde concentrations may exceed a certain threshold and function as a diffusible bloom termination signal that leads to population

cell death (Vardi et al. 1999; Bidle and Falkowski 2004; Casotti et al. 2005). Therefore decadienal-like aldehydes may not only affect the reproductive capacity of grazers but also act as infochemicals to provide a surveillance system to evaluate stress during phytoplankton blooms. It is now established that coordination of stress responses, cell survival, and death, as observed in multicellular organisms, can also operate at single cell level (Ameisen 2002).

Since there are species-specific differences in sensitivity to 2,4-decadienal, as observed between *Phaeodactylum tricornutum* and *Thalassosira weissflogii* showing an EC_{50} for growth of 7.1 µmol L^{-1} and 1.9 µmol L^{-1}, respectively (Vardi et al. 2006; Casotti et al. 2005), Vardi et al. (2006) proposed that the differential production and sensitivity to reactive aldehydes may determine the fitness and succession of phytoplankton communities in the marine environment. However, precautions have to be taken since *P. tricornutum* does not produce 2,4-decadienal (Wichard et al. 2005a) and is also little represented in natural ecosystems. Preliminary experiments on PUA-producing *Skeletonema marinoi* did not reveal a resistance system when exposed to self-produced 2,4-octadienal and neither to 2,4-decadienal (data not shown). Therefore, it is possible that such an effect is species-specific and/or takes place under very special circumstances.

Nevertheless, the evidence that diatoms use PUAs as chemical signaling compounds provides new insights into the cellular mechanisms mediating biotic

interactions at the population level and challenges traditional concepts of phytoplankton bloom dynamics. With hundreds of diatom species producing potentially a diverse array of aldehydes and other oxylipins, these reactive compounds may prove to be even more versatile than shown here , and may be very likely a key factor contributing to the ecological success of these organisms.

CHAPTER 4

Patterns of cell lysis in the diatom *Skeletonema marinoi* in batch cultures and in natural populations

I. General introduction

Cell death and subsequent lysis of phytoplankton have been shown to result from a number of factors, including attack by viruses, or exposure to physiological extremes of light, temperature and nutrient concentration (Kirchman 1999). Despite the potential importance of factors leading to cell lysis, this process is largely neglected in efforts to account for phytoplankton losses in the sea. The main reason for the paucity of our knowledge on phytoplankton lysis rates in nature is the lack of reliable techniques to quantify this process (Franklin et al. 2006).

The first attempt to measure phytoplankton cell lysis in a natural system was made fifteen years ago. Van Boekel et al. (1992) introduced a method based on the measurement of dissolved esterase activities (DEA) to obtain a semi-quantitative estimate of cell lysis rates in a *Phaeocystis* bloom in the North Sea. Dissolved esterases are enzymes present in the cytoplasm of living cells; when they are found free in the water, they are assumed to result from cell breakage or membrane damage

and could therefore serve as tracers of cell lysis. The method relies on cleavage of fluorescein diacetate (FDA), which is added as a substrate in excess, yielding the free fluorescein, which is highly fluorescent. Fluorescein accumulates in samples as a product of the reaction between esterases and FDA, so that the resulting fluorescence intensity is proportional to the esterase activity. The fluorescence is converted into fluorescein concentration either using a conversion factor based on a calibration curve (Van Boekel et al. 1992), or by using an internal standard that is added immediately after the measurement (Riegman et al. 2002). The cell lysis rate is calculated as the decrease in particulate esterase activity (PEA) with time due to the production of DEA during cell lysis (produced on an hourly basis):

$$\mu\,(\mathrm{d}^{-1}) = \frac{\mathrm{DEAprod}}{\mathrm{PEA}} \times 24 \qquad (1)$$

$$\text{where } \mathrm{DEAprod} = \frac{\mathrm{DEA}}{T_{1/2}} \times 0.5 \qquad (2)$$

and $T_{1/2}$ represents the turnover time of the naturally-occurring DEA, measured after 24 h of incubation.

The estimation of PEA is a critical point in the esterase method. Due to potential contributions to PEA from other planktonic organisms (bacteria, zooplankters) not relevant to the measurements, it has been proposed that estimates of phytoplankton PEA be based on Chl *a* concentration in the field samples using an esterase

activity/Chl *a* ratio derived from exponentially growing algae in batch cultures. Both van Bockel (1992) and Agusti et al. (1998) used this approach to derive the PEA concentrations of the ambient phytoplankton population. However, the two estimates varied widely between these two studies (13 and 335 nmol FDA h^{-1} µg Chl a^{-1}, respectively). Indeed, Berman and Wynne (2005) demonstrated that PEA Chl^{-1} varied widely in freshwater algae in culture, not only between different algal species, but also due to different stages in the growth of the same organism. Furthermore, there appeared to be no general pattern of PEA Chl a^{-1} change with growth phase in their study. Because of the high variability of this parameter, Riegman et al. (2002) and Berman and Wynne (2005) proposed using direct measurements of PEA, by adding FDA to untreated samples for the calculation of lysis rate. This may lead to an underestimation of PEA (and therefore an overestimation of the lysis rate), since Agusti et al. (1998) observe that the fluorescein fluorescence emitted by the phytoplankton cell depends on both the size of these cells and their internal concentration in esterases; so that the fluorescence of small phytoplankton cells may be lower than bigger cells with similar esterase content (the so-called "packaging" effect).

Another factor to take into account is spontaneous FDA hydrolysis, due to the instability of FDA in seawater. Riegman et al. (2002) modified the esterase method by correcting all measurements of esterase activity by the spontaneous hydrolysis of FDA, referred as the non-enzymatic hydrolysis of FDA. This parameter is measured by passing the sample through <10 kDa centrifuge filter device, which remove all

enzyme protein (see Berman and Wynne 2005). Such a correction resulted in lysis rate estimates lower from those calculated previously, and is especially important when the esterase activity was low.

An important point of divergence between the different authors concerned the calculation of the lysis rate. Van Boekel et al. (1992) used linear kinetics assuming that the release of DEA, in terms of absolute amount, is linear with time (see equation 1). Linear rates would be appropriate if the esterase activity within phytoplankton cells and esterase decay rate are in steady state within the time interval involved in the rate calculation (i.e., one day). This implies that during the decay of a population, a linearly increasing fraction of the population is senescent. Agusti et al. (1998) assumed that phytoplankton mortality followed exponential kinetics, since (2) during bloom collapse, phytoplankton biomass decreases rapidly over time (Brussaard et al. 1995) and (2) mortality are commonly accepted as exponential processes in general demographic analyses in terrestrial (Sarukhan et al. 1985) and marine plants (Duarte et al. 1994). However it is important to stress that there is no evidence available, so far, to support either exponential or linear approach for phytoplankton mortality. The use of an exponential model to calculate phytoplankton lysis rates has the advantage of allowing direct comparison with growth rates, which are commonly assumed as exponential rates. Therefore Agusti et al. (1998) calculate lysis rates as following:

$$\mu\,(d^{-1}) = -\ln\frac{PEA - DEAprod}{PEA} \times 24 \qquad (4)$$

where DEA prod is calculated as equation 2.

In both methods of calculation, lysis rates are expressed in d^{-1} by multiplying by 24, assuming that the esterase activity and decay rates are constant over a 24-h period. However, although the lysis rate, calculated on an hourly basis, may correspond to the actual rate of lysis at the time of sampling, it is uncertain if these rates should be extrapolated to a daily period merely by multiplying by 24. Berman and Wynne (2005) proposed that the method should be modified by more frequent measurements of ambient PEA and DEA over a 24-h period to improve its reliability as a quantitative measure of daily lysis rate.

The esterase method in its present form has the advantage of being simple and non-invasive. However, the major problem that remains is that the term "esterase" is generic to any enzyme that catalyses the hydrolysis of organic ester compounds. A large number of enzymes can function as esterases, such as lipases, acetylesterases, nucleases, phosphatases, aminopeptidases and proteases, and are ubiquitous in all domain of life (Sharma et al. 2001; Gilham and Lehner 2005).

Bacteria produce a wide range of extracellular enzymes, such as leucine-aminopeptidases in marine (Martinez and Azam 1993) and freshwater systems (Sinsabaugh and Foreman 2001) and the activity of these enzymes increases at the end of phytoplankton blooms (Gajewski and Chrost 1995). Reports have shown that the dissolved esterase activity increases at the end of blooms (van Boekel et al. 1992; Brussaard et al. 1995; Agusti and Duarte 2000), but the ability of extracellular esterase

enzymes from bacteria to cleave FDA, such as leucine-aminopeptidase, could potentially confound the estimation of dissolved esterase activity from phytoplankton cells and therefore overestimate phytoplankton lysis rates.

It is clear that bacteria can cleave FDA (FDA hydrolysis is a common method to estimate microbial activity) (Schnürer and Rosswall 1982), but their impact on the estimation of esterase activity from the phytoplankton has been dismissed by the users of the esterase method. In both marine and freshwater systems, the presence of 1-3 x 10^6 bacteria mL^{-1} in an algal culture increased the esterase activity by less than 5% (Van Boekel et al. 1992; Berman and Wynne 2005). Despite the fact that the metabolic state of the bacteria was not determined, these authors conclude that bacteria do not significantly bias in the determination of PEA. Agusti et al. (1998) tested the capacity of bacterial esterases to cleave FDA by lysing bacterial cells and measuring FDA cleavage. Bacterial cells showed 10-50 fold less esterase activity than phytoplanktonic cells of similar size, but in this case, they did not quantify the bacterial biomass or the metabolic state at their sampling sites. Nevertheless, these authors assumed that, by far, the main contribution of DEA was derived from phytoplankton with only a minor contribution from heterotrophic plankton.

One of the major limitations in the study of cell lysis is that, once cells have lost their pigments, they tend to disintegrate fully to become unrecognizable detritus. Only phytoplankters with more solid cell-walls, such as silica frustules in diatoms, leave recognizable structures that remain after deterioration. Therefore the presence of

empty silica cells has been used to estimate the number of dead diatoms (Reynolds and Wiseman 1982). This method, restricted to diatoms, may underestimate the death rate because irreversible loss of physiology activity and structural damage can occur prior to complete structural disintegration.

Another method has been developed by Baldi et al. (1997) to estimate *in situ* phytoplankton cell lysis, based on the estimation of extracellular lipid and polysaccharide composition. However, this method requires the analysis of the complete lipid class distribution (which limits the number of samples that can be analyzed) and does not give any indication about the rate of cell lysis.

Garcès and Maso (2001) proposed a method based on the concurrent measurements of growth rate by cell-cycle analysis and cell number increase. Cell-cycle analysis defines the potential growth rate without considering cell mortality, whereas changes in cell number takes into account the loss of phytoplankton cells, so the difference between the two approaches indicates cell mortality. However, this method may be often biased by the underestimation of the *in situ* growth rate based on number of cells (e.g. natural population may divide actively *in situ* but not in a bottle) (Chang and Carpenter 1991) and this may result in an overestimation.

Alternative techniques based on vital fluorescent stains, such as SYTOX Green nucleic acid stain, which only penetrates cells with compromised cell membranes (Roth et al. 1997), are commonly used in culture to estimate the percentage of dead cells (Veldhuis et al. 1997, 2001). The SYTOX Green labels the nucleus a brilliant green, but does not stain if lysis occurs and cell contents are lost. Therefore this method is only useful to detect an early stage of cell death. In addition, the SYTOX

Green method is difficult to apply to natural phytoplankton communities, since the degree of staining varies considerable among the different species and pigments interfere with the fluorescent signal of the stain, thereby leading to ambiguity in differentiating stained and unstained cells (Veldhuis et al. 2001).

More recently, Agusti and Carmen-Sanchez (2002) developed a method based on the enzymatic digestion of dead cells by DNAse and trypsin. Living cells in a phytoplankton population are defined as those that survive the digestion procedure. This method is strongly dependant on the accuracy of the cell counts, and the authors recommend the use of a flow cytometer. Here again, cells that have "lost their pigment content" are not taken into account, since the detection of phytoplankton cells by flow cytometry is usually based on the presence of chlorophyll pigment. In addition, the configuration of most commercially available flow cytometers is best suited to cells varying in size between 0.5 and 30 μm (Veldhuis and Kraay 2000), and therefore the method of Agusti and Carmen-Sanchez (2002) may be difficult to apply for larger phytoplankton cells and those that form long chain and aggregates.

II. Estimation of lysis rates in batch cultures of *Skeletonema marinoi*

II.1. Introduction

In order to validate lysis rate estimates, the esterase method was applied to the species *Skeletonema marinoi* Sarno & Zingone, which is responsible for late winter blooms in the Northern Adriatic Sea. Initially, lysis rates were measured in axenic monocultures (isolated from the Northern Adriatic Sea) using an improved version of the esterase method (Riegman et al. 2002). To determine whether DEA was correlated with cell lysis, lysis rates were calculated during the growth cycle and were compared with two others methods, SYTOX Green staining (Veldhuis et al. 2001) and enzymatic digestion (Agusti and Carmen-Sanchez 2002), which both target only cells with permeable cell membranes. In addition, esterase activity has been quantified using the esterase method over a 24-h period every 3 hours under laboratory conditions in order to verify the stability of esterase activity during the diel cycle. Once the best protocol was determined, this was applied *in situ* during *Skeletonema* blooms in the Northern Adriatic Sea.

II.2. Materials and Methods

II.2.1 Cultures

An axenic culture of *Skeletonema marinoi* Sarno & Zingone (Bacillariophyceae) (formerly *S. costatum*), originally isolated from the North Adriatic Sea, was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton, West Boothbay Harbour, ME, USA (strain CCMP 2092). The diatom was cultured in a 2-liter polycarbonate bottle with an air bubbling system (Nalgene) in a thermostated environmental chamber (Hereaeus, Germany). The culture was maintained at 17°C on a 12h-12h light-dark cycle under a photon flux density of 150 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes (Phillips TLD 36W/950). *S. marinoi* was grown in one-month-old natural seawater amended with f/2 medium nutrients (Guillard 1975), except for nitrate (145 µmol L^{-1}), which was lower in order to reach the declining phase faster. Cell concentrations were monitored daily by flow cytometry (FACScalibur, Becton Dickinson), equipped with standard optics (see Chap II-B).

Samples were taken 6 h after the onset of the light period, in order to avoid interference from potential circadian variability, except for the diel variability tests, when the cultures were sampled every 3 hours.

II.2.2 The FDA method

PEA was determined directly on untreated samples and DEA on 0.7 µm filtrates (GF/F glass filter, Whatman). The nonenzymatic hydrolysis of Fluorescein-DiAcetate (FDA, Sigma Aldrich) was determined by first removing esterases from the sample, using <10 kDa centrifuge filters (Nalgene Midi Centrifuge Filter, 10 K MWCO, PES; 4.0 mL, Nalgene). All esterase activity measurements were performed under identical standard conditions: 0.1 mL of Tris–HCl (pH 8.0; final concentrations 0.5 mmol L^{-1}) + 50 µl EDTA (final concentrations 0.5 mmol L^{-1}) and 20 µl of FDA (in acetone; final concentration 20 µmol L^{-1}), were added at time t = 0, into 1.9 mL of sample. The increase in fluorescence, at 520 nm, of the samples was recorded following an incubation at 20 °C for 60 min. Fluorescence was converted into fluorescein concentration using an internal standard (Fluorescein, Sigma, final concentration 5 nmol L^{-1}) that was added immediately to the sample after the measurement at time t = 60 min.

Production rate of DEA in 1 h was calculated following equation 2.

Turnover-time ($T_{1/2}$ in h) of natural DEA was determined at t = 0 and after 24 h incubation of a 0.7 µm filtrate at ambient temperature in the dark.

Phytoplankton lysis rate was calculated following equation 1 (linear model).

Percentage of dead cells was calculated by dividing DEA by the total amount of esterase activity (PEA + DEA).

As noted by both Riegman et al. (2002) and Agusti and Duarte (2002), the measurement of PEA is a critical factor in the esterase method directly affecting the estimation of lysis rate. Therefore two methods were tested for the estimation of PEA: (1) Sonication. Cells were sonicated for 30 sec, 1, 5 and 10 minutes in a 0.5 liter-water bath (Ultrasonik Tabletop Cleaner, NEY). (2) Direct addition of FDA to untreated samples. PEA was determined under standard conditions (see above). Since PEA extraction efficiency may also depend on the algae cell concentrations (Agusti et al. 1998; Agusti and Duarte 2002), samples were taken from cultures in the early stationary phase.

The measurement of PEA in untreated samples may be underestimated due to a possible packaging effect (i. e. considering two cells with the same amount of PEA, the fluorescence of the fluorescein may be lower in a small cell with respect to a bigger one because of the reabsorption of fluorescence by close-by molecules). To test this in *Skeletonema* cultures, 10 mL of cultures in early stationary phase were incubated with FDA as described above. After incubation, cells were harvested by filtering onto 0.7 μm glass filters (GF/F Whatman), resuspended with Tris-HCl + EDTA (pH 8.0; final concentration 0.5 mmol L^{-1}) and 2 mL aliquot were sonicated for 5 minutes. Fluorescence was determined before and after sonication.

For chlorophyll *a* concentrations, 30 mL of cultures were filtered through 25-mm GF/F filters (Whatman) and immediately frozen in liquid nitrogen. Chl *a* concentrations were measured spectrophotometrically after 90% acetone extraction for

2 h at room temperature followed by 20 min centrifugation at 3000 g (Jeffrey and Humphrey 1975). Chl *a* concentrations were calculated as:

$$\text{Chl } a\ (\mu g\ L^{-1}) = \frac{V_{extract}}{V_{filtered}} \times (11.47\ \lambda_{664} - 0.4\ \lambda_{630}) \qquad (6)$$

Where λ_{664} and λ_{630} represent the absorption at 664 nm (for Chl *a* peak) and 630 nm (for Chl *c* peak), respectively, and $V_{extract}$ and $V_{filtered}$ are the volume of extract and the volume filtered (in mL) (Jeffrey and Humphrey 1975).

II.2.3 SYTOX Green staining method

The test is based on the staining of dead cells using the vital SYTOX Green Nucleic Acid Stain (Molecular Probes). This stain is used to determine cell viability in phytoplankton (Veldhuis et al. 2001) and bacteria (Lebaron et al. 1998). The assay is based on the fact that the stain only penetrates cells with compromised cell membranes but will not cross membranes of living cells (Roth et al. 1997). Thus, discrimination between living and dead cells is based on the different green fluorescence intensity: non-viable cells will have high green fluorescence whereas viable cells will not stain significantly. Optimal final concentration (500 nmol L^{-1}) and time of incubation (10 min) were previously determined experimentally for *S. marinoi* (see Chap III-B). The

percentage of dead cells is calculated by dividing the number of cells with high green fluorescence from the total cell number.

Lysis rates were calculated following Brussaard et al. (1997):

$$\mu\,(\mathrm{d}^{-1}) = (\ln X_t - \ln X_0) \times \left(\frac{Y_t - Y_0}{(X+Y)_t - (X+Y)_0} - 1 \right) \qquad (7)$$

where $(x+y)_t$ represents the total concentrations of cells at time t, and x_t and y_t the concentration of living and dead cells at time t, respectively.

II.2.4 The enzymatic digestion method

This method is based on the brief exposure of the cells to the enzymes trypsin and DNAse I, which enter the cytoplasm of cells with damaged plasma membranes, resulting in the digestion of the cells (Agusti and Carmen-Sanchez 2002). Stock solutions of DNAse I (800 μg mL^{-1}, Sigma Aldrich) and trypsin (2%, Sigma Aldrich) were prepared in Hanks Balanced Salt medium (HBSS, Sigma Aldrich) without red phenol and kept frozen at -20 °C until use. DNAse I (final concentration of 120 μg mL^{-1}) was added to the sample and followed by 15 min- incubation at 37°C, and then trypsin was added (final concentrations 3 mg mL^{-1}) for an additional 30 min- incubation at 37°C. At the end of the incubations, samples were placed on ice in order to stop the reaction. Samples used as blanks were incubated in the same conditions

using only HBSS medium instead of the enzyme cocktail. For all experiments, the potential impact of ice incubation on cell counts was verified by counting the same sample at the beginning and end of the experiment. Cell counts were determined by flow cytometry (FACSCalibur, Beckton Dickinson). 3.7-μm diameter beads (Coulter Flowset Fluorospheres) were used as internal standards. Experiments were performed from three independent experiments and with methodological triplicates for each experiment.

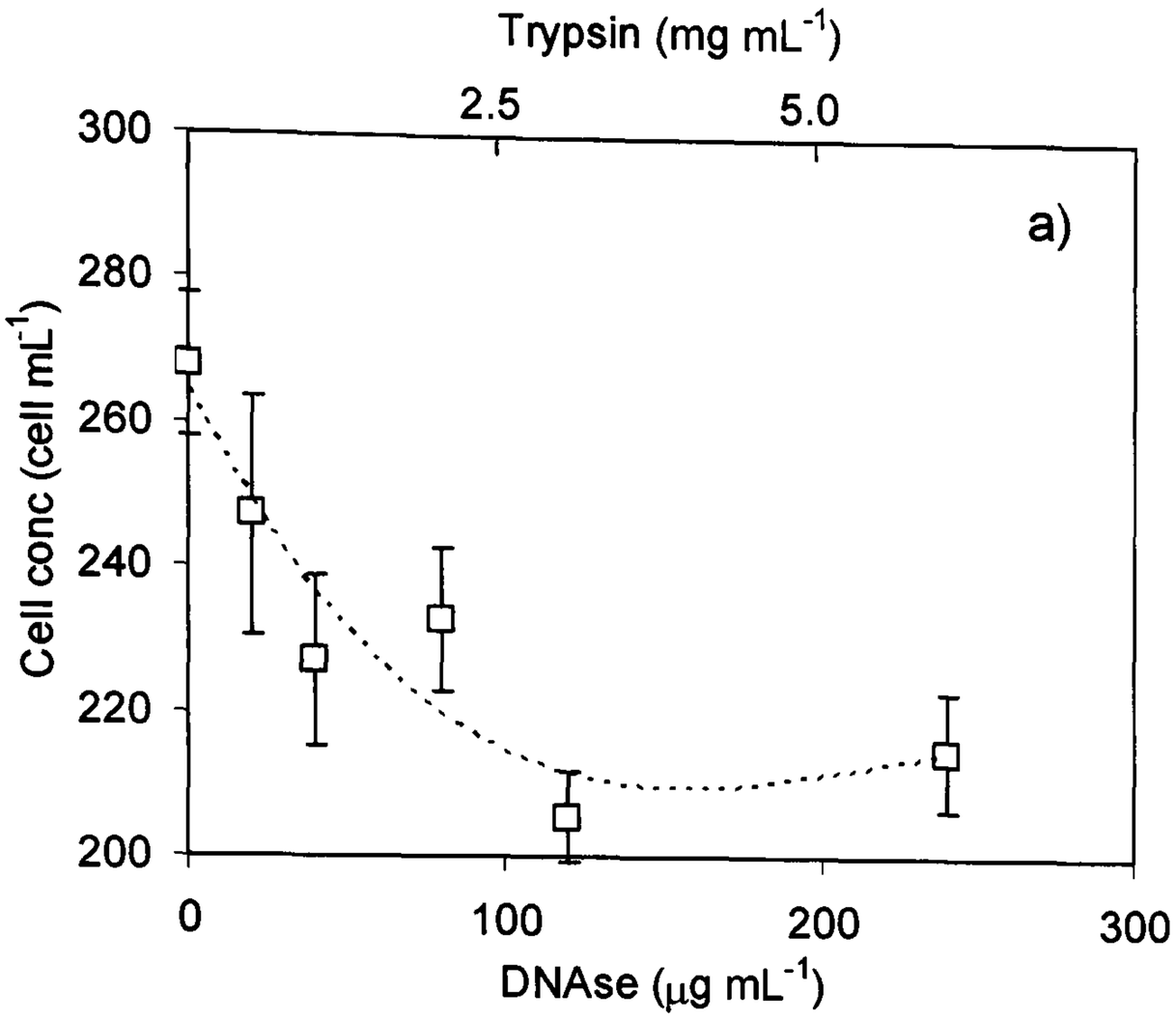
The percentage of viable cells in the culture was calculated by dividing the concentration of viable cells after enzyme treatment by the cell concentration of the blanks, which represent the total cell concentration. The number of dead cells was calculated by subtracting the number of living cells from the total cell abundance. Lysis rates were calculated using equation (7) (see above).

The effects of incubation temperature in the efficiency of the digestion of dead cells were tested by incubating duplicate samples of *S. marinoi* culture in declining phase of growth at 37°C or 20°C. The digestion procedure was more effective at 37°C than at 20°C, with 23.5% (± 4.1% SD) and 11.9% (± 7.3% SD) of digested cells, respectively. Therefore, 37°C incubation temperature was used thereafter in order to quantify lysis rates during the growth cycle.

The effect of incubation times in the efficiency of digestion was investigated by testing different incubation times from 45 min to 90 and 120 min at 37°C of triplicate samples from *S. marinoi* culture in the declining phase of growth. The concentration of digested cells (dead cells) was not affected by incubation time: 23.5%, 27.0% and

25.5% of digested cells were found after 45, 90 and 120 min incubation, respectively. Since differences between the three incubation times were not significant, 45 min incubation was used thereafter.

The effect of enzyme concentration on the digestion of dead cells was tested by adding different concentrations of DNAse I and trypsin to the samples. Five final concentrations of DNAse I (0, 20, 40, 80, 120 and 240 μg mL^{-1}) and trypsin (0, 0.38, 0.75, 1.5, 3 and 6 mg mL^{-1}) were tested in 1 mL aliquots of duplicate cultures, which were then processed after 45 min incubation at 37°C. Tests were conducted with cultures in stationary and declining growth phase. Cell concentrations after enzymatic digestion decreased asymptotically with increasing enzyme concentration to reach a plateau at 3 and 1 mg trypsin mL^{-1} and 120 and 40 μg DNAse mL^{-1} in stationary and declining phase, respectively (Figure IV-1). Therefore, final concentrations of DNAse I and trypsin of 120 μg mL^{-1} and 3 mg mL^{-1}, respectively, were used thereafter.



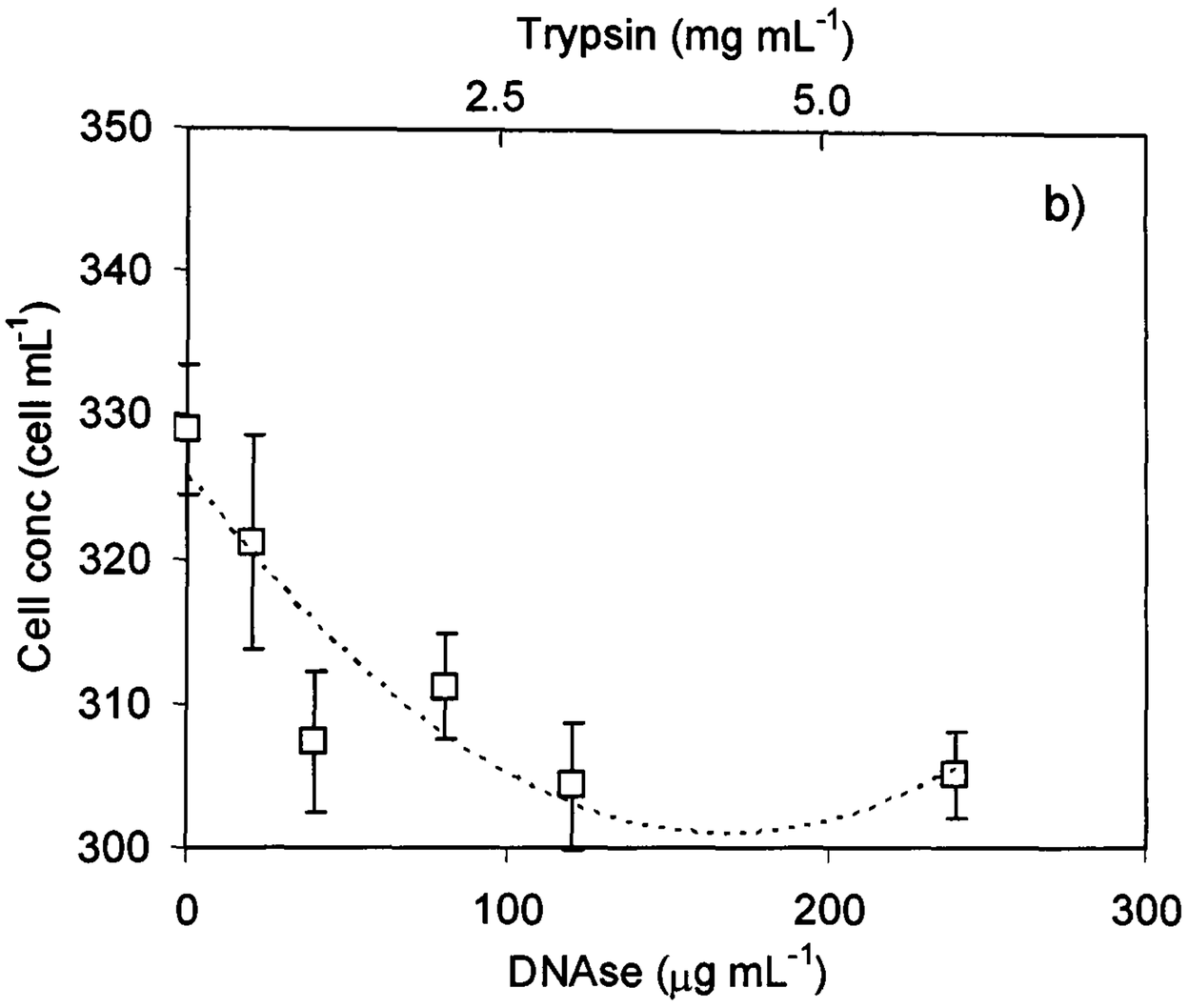


Figure IV-1. The effect of a variable concentration of the enzymes (DNAse I and Trypsin) on the number of livings cells remaining after the cell digestion assay in cultures of *Skeletoenema marinoi* grown a) in the stationary b) and the declining phase. Data are means of technical duplicates from a single experiment. Error bars are standard deviations.

II.3. Results and Discussion

II.3.1 Optimization of PEA measurements

PEA in sonicated samples in early stationary phase corresponded to 2602 (± 48 SD), 2552 (± 58 SD), 2745 (± 100 SD), 2882 (± 134 SD) and 1533 (± 265SD) nmol FDA h^{-1} L^{-1} after 30s, 1, 5 min and 10 min sonication (Figure IV-2). Only 10 minutes sonication induced a significant effect on PEA (Dunnett Multiple Comparison post-test, $p < 0.01$) with a reduction of 41 % of the esterase activity. This result indicates a potential degradation of the esterase by sonication, which is in agreement with Riegman et al. (2002) who noticed an instantaneous and high loss of esterase activity following cell sonication. Therefore, PEA of natural phytoplankton population was estimated by direct addition of FDA into untreated samples.

II.3.2 Testing the "packaging" effect

In order to determine the underestimation of PEA in untreated cells caused by a possible packaging effect, cells resuspended in the lysis buffer after the incubation were sonicated. PEA reached 226 ± 33 nmol FDA h^{-1} L^{-1} in sonicated samples and 210 ± 22 nmol FDA h^{-1} L^{-1} in untreated samples and values were not significantly different (Student *t*-test, $p > 0.05$).

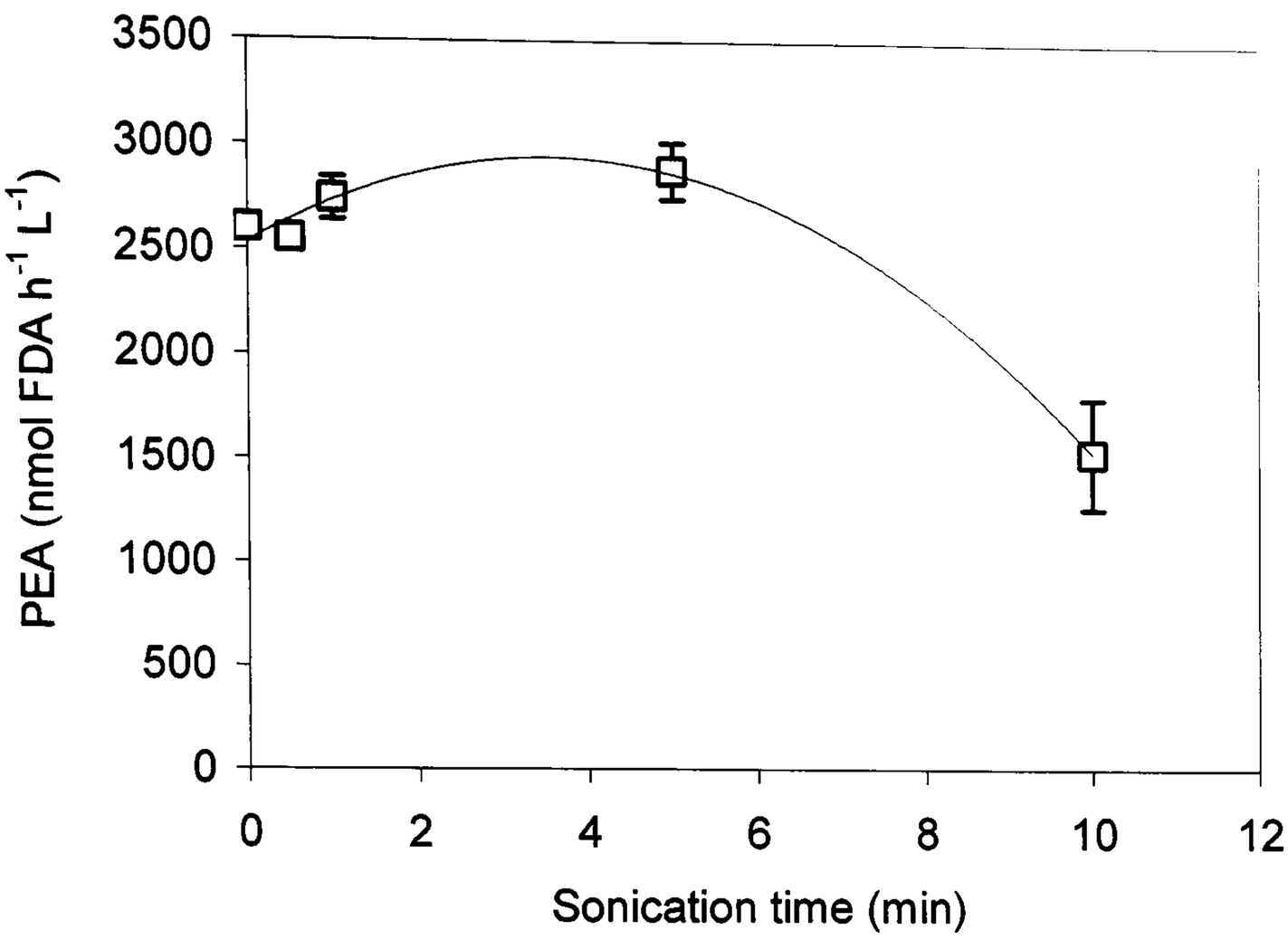


Figure IV-2. Particulate esterase activity (PEA in nmol FDA h^{-1} L^{-1}) of *Skeletonema marinoi* sampled in the early stationary phase, after different sonication times (min). Data are means of replicates from 3 independent experiments. Error bars are standard deviations

In addition, since only 11 % of the produced fluorescein was present in the cells after 1 h of incubation (see above), the "packaging" effect would represent an underestimation of PEA of less than 1%, which is not statistically significant (Student *t*-test, $p > 0.05$). Riegman et al. (2002) reached the same conclusion. In addition, when applied to natural blooms of *Skeletonema marinoi*, it must be considered that cells are often bigger than in culture (Raffaella Casotti, pers. comm.), which indicates the possible "packaging" effect may be negligible.

II.3.3 Testing the stability of esterase activity during a 24h-diel cycle

A 24 h time-course experiment was performed using a culture of *Skeletonema marinoi* in the declining phase of growth. Esterase activity and percentage of SYTOX Green-positive cells were quantified every three hours in order to test the stability of esterase activity during a diel cycle. PEA values as well as PEA per cell did not vary significantly during the 24-h period (Bonferroni's multiple comparison test, $p>0.05$,) (Figure IV-3). DEA was significantly different only at two time points (t 3 h and t 24 h) (Bonferroni's multiple comparison test, $p<0.05$) while percentages of dead cells were never statistically different (ANOVA, $p>0.05$) (data not shown). It is then possible to conclude that esterase activity remains stable over a 24-h period at least for *S. marinoi*, and therefore extrapolation to a daily period for the calculation of the lysis rate could be applied, as proposed by Agusti et al. (1998).

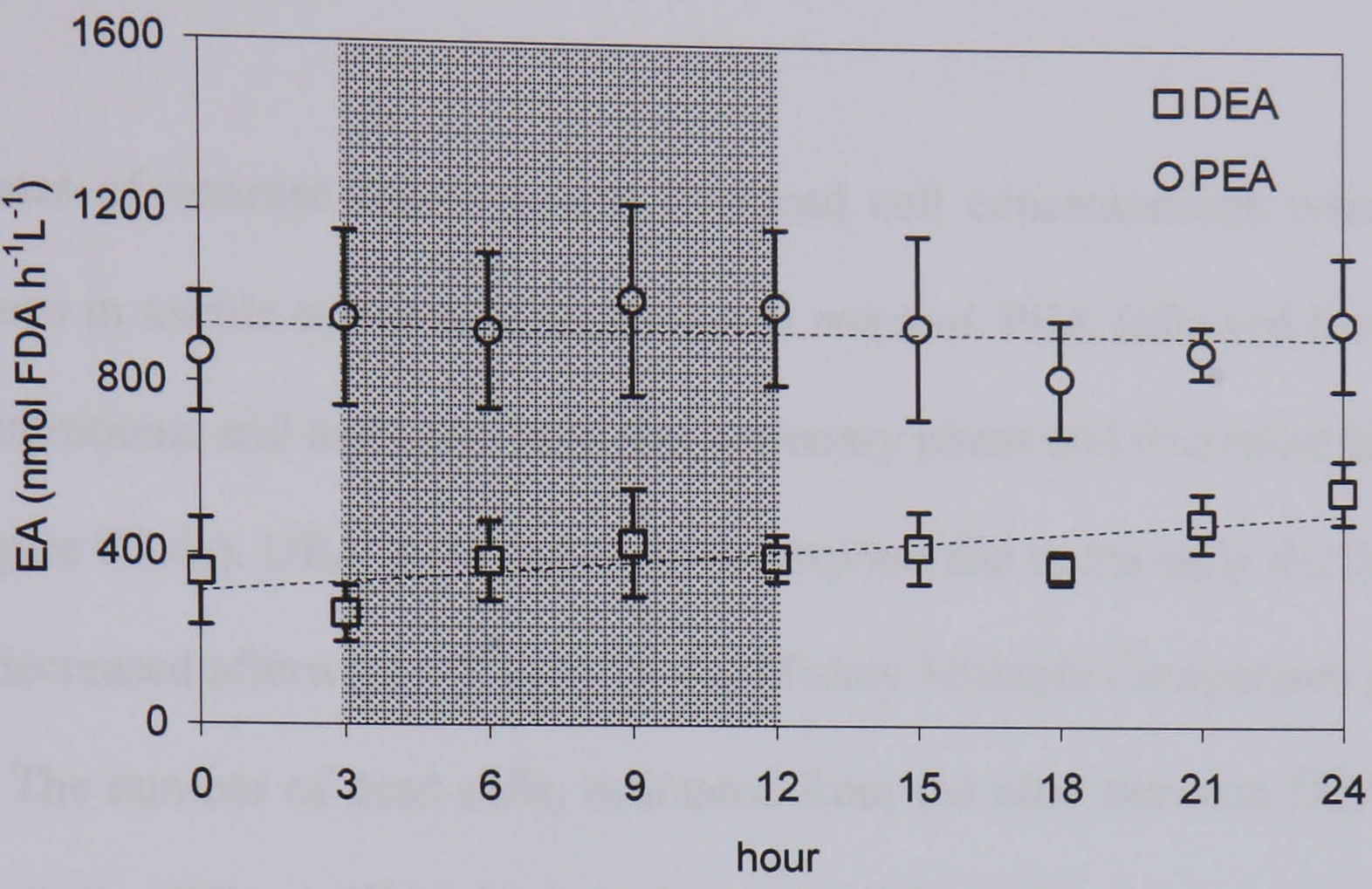


Figure IV-3. Dissolved esterase activity (DEA in nmol FDA h^{-1} L^{-1}) and particulate esterase activity (PEA in nmol FDA h^{-1} L^{-1}) over a 24h-period in a culture of *Skeletonema marinoi* during the declining phase. Shaded region represents dark period. For all plots, data are means of replicates from 3 independent experiments. Error bars are standard deviations.

II.3.4 Variation of esterase activities during the growth cycle

Estimates of esterase activities and live/dead cell concentrations were followed over 16 days in axenic cultures of *Skeletonema marinoi*. PEA followed the pattern of cell concentrations, and increased until the stationary phase and decreased in declining phase (Figure IV-4a). DEA increased from the exponential to the early declining phase and then decreased afterwards (Figure IV-4a) (Tukey Multiple Comparison post-test, $p < 0.001$). The number of dead cells, estimated from the ratio between DEA and total esterase activity (DEA + PEA), increased exponentially during the growth cycle ($R^2 = 0.94$) (Figure IV-4b).

Esterases have so far only been found as intracellular enzymes in phytoplankton cells and are not produced extracellularly (Chrost 1991); therefore their presence in water can be used as an indicator of cell lysis. The percentage of dead cells estimated from esterase activity followed the same patterns as those obtained with the SYTOX Green and enzymatic digestion methods, which indicates that the concentration of dissolved esterases is correlated with the number of dead cells estimated by the two other methods. In addition, no bacteria were present in the culture (checked at the beginning and at the end of each experiment), so the contribution of bacteria to esterase activity can be excluded. This result indicates that dissolved esterases originated only from the lysis of diatom cells and that esterase activity can be used as an indicator of phytoplankton cell lysis.

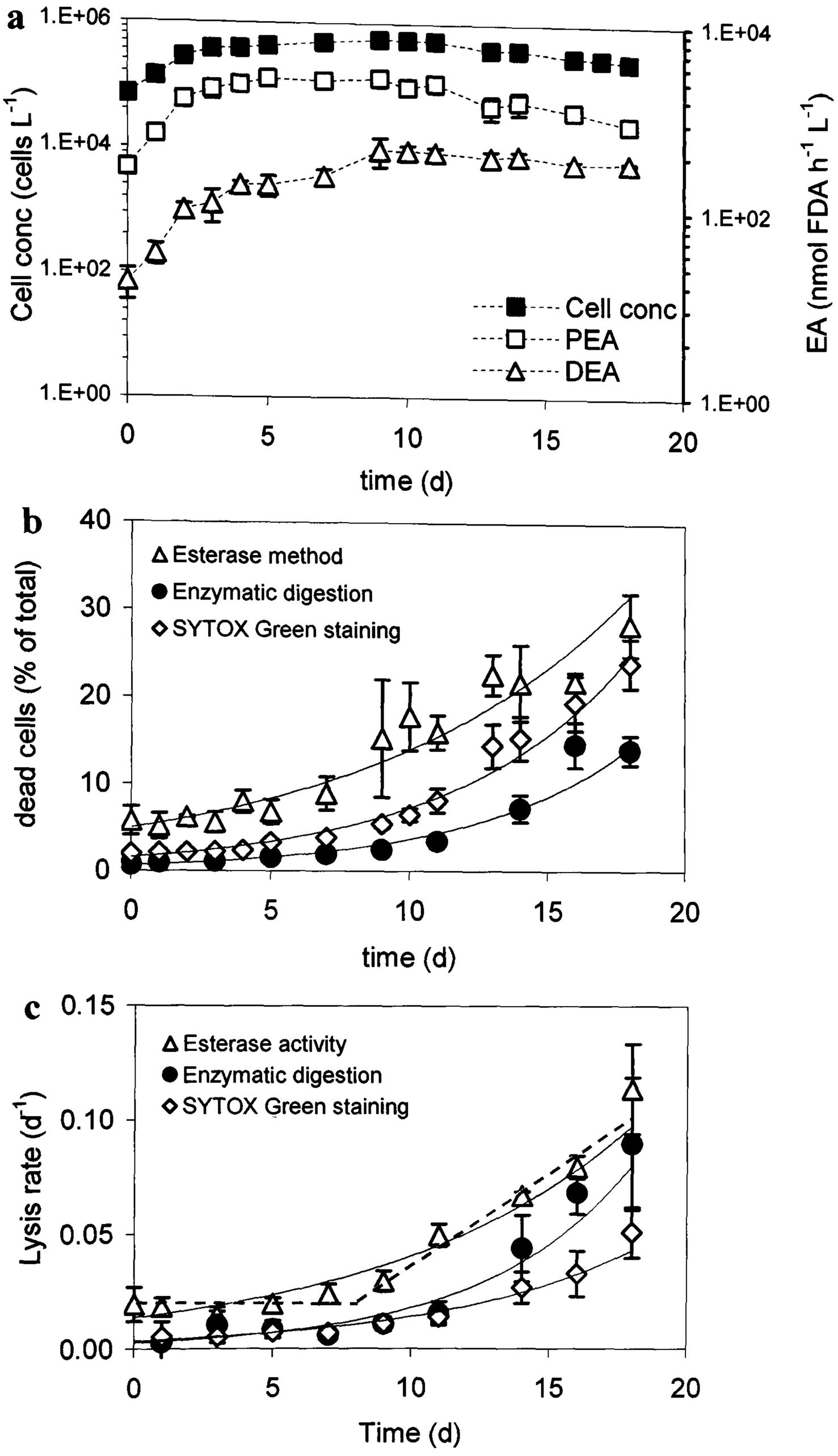


Figure IV-4. a) cell density (cells L^{-1}), particulate esterase activity (nmol FDA h^{-1} L^{-1}) and dissolved esterase activity (nmol FDA h^{-1} L^{-1}) in *Skeletonema marinoi* cultures. b and c) Percentage of dead cells and lysis rate estimated by esterase activity, enzymatic digestion and SYTOX staining method. For all plots, data are means of replicates from 6 independent experiments. Error bars are standard deviations.

Lysis rates estimated by the three methods remained very low in the exponential phase and started to increase in the late stationary – early declining phase of growth (Figure IV-4c). The pattern was similar among the three methods; diatom cell lysis appeared to follow an exponential kinetics during the growth cycle (Figure IV-4c), suggesting that lysis rate may be better calculated using the exponential rather than the linear model (equation 3 and 1 Chap IV-A, respectively), as suggested by Agusti et al. (1998). However, if only the period of the increase of cell death is considered (from late-stationary phase), it becomes less clear which of the linear (Figure IV-4c, red dashed) or exponential (Figure IV-4c, black dashed) models better fit the kinetics of cell lysis.

The values of lysis rates calculated with equation 1 (linear model) were 0.01% lower than those calculated with equation 4 (exponential model) (Student t-test, $p < 0.05$), so the choice of the model has only a minor impact on our lysis rate estimates. Since there is no evidence, as yet, to support either approach in phytoplankton mortality, and values calculated with equation 1 are slightly lower (and therefore are less prone to overestimation), linear model was applied to calculate lysis rates at sea, as used by Van Boekel et al. (1992), Riegman et al. (2002) and Riegman and Winter 2003).

The percentage of dead cells estimated by the esterase method are relatively higher than those obtained with the SYTOX Green staining and the enzymatic digestion methods; the latter giving the lowest values (Fig. IV-4b). Despite the fact that these methods all target the process of cell membrane permeabilization, its quantification

differs radically. The SYTOX Green staining method is based on the labeling of dead cells among the cell population using a single sample, while the enzymatic digestion method quantifies the total cell number in the population in one sample, and the number of viable cells in another one, to infer the percentage of dead cells. The higher percentage of dead cells with the SYTOX Green staining with respect to the enzymatic digestion method may indicate that SYTOX Green is able to penetrate cells in an earlier stage of cell membrane permeabilization than the enzymes (DNAse I and Trypsin). Since in our case both methods relied on flow cytometry for cell counts, which is based on chlorophyll fluorescence, these two methods did no take into account empty cells The presence of empty cells is confirmed in cultures of *Skeletonema* during the declining phase of growth (data not shown) and in natural assemblages of phytoplankton (e.g. Baldi et al. 1997). Therefore, SYTOX Green staining and enzymatic digestion methods can only be used to detect early stages of cell death.

In contrast, the esterase method is based on the release of intracellular content and therefore considers all stages of cell death, from the beginning of cell membrane permeabilization until the complete release of cell contents. This may explain the higher percentage of dead cells and lysis rates obtained with respect to the two other methods. Since the measurement of esterase activity is a non-invasive method, i. e. the treatment does not induce cell lysis, the higher values should be real and not an artefact. Therefore the quantification of esterase activity is a more sensitive technique to estimate cell lysis in cultures that the two other methods. Despite that the esterase method is prone to biases from the origin of the esterases and perhaps to

overestimates, the quantification of esterase activity offers an attractive technique to study phytoplankton cell lysis at sea.

PEA $cell^{-1}$ increased during exponential phase and reached the maximum of 5.94 ± 0.49 fmol FDA h^{-1} $cell^{-1}$ in the early stationary phase and then it decreased progressively during the declining phase, reaching 3.38 ± 0.77 fmol FDA h^{-1} $cell^{-1}$ at the end of the experiment (Figure IV-5). In early stationary phase, cell volume was estimated to be 55.7 ± 20.8 μm^3 (determined by microscopy, considering *S. marinoi* as a cylinder) leading to a cell esterase activity per unit volume of 0.10 fmol FDA h^{-1} μm^{-3}. This result is consistent with the esterase activity of two other diatoms *Phaeodactylum tricornutum* and *Chaetoceros decipiens* which produced 0.51 and 0.05 fmol FDA h^{-1} μm^{-3} in the exponential phase (Agusti et al. 1998). The variability of PEA $cell^{-1}$ may very likely depend on cell volume, as found by Agusti et al. (1998).

Esterase decay rate was measured every three days after 24 h at 17 °C in the dark (Figure IV-6). No difference was observed during the growth cycle (ANOVA, $p>0.05$). The average of the esterase half-life time (42.2 ± 12.6 d^{-1}) is in remarkably good agreement with the half-life time of natural phytoplankton esterase (49 ± 6 d^{-1}) incubated at 15°C (Riegman et al. 2002).

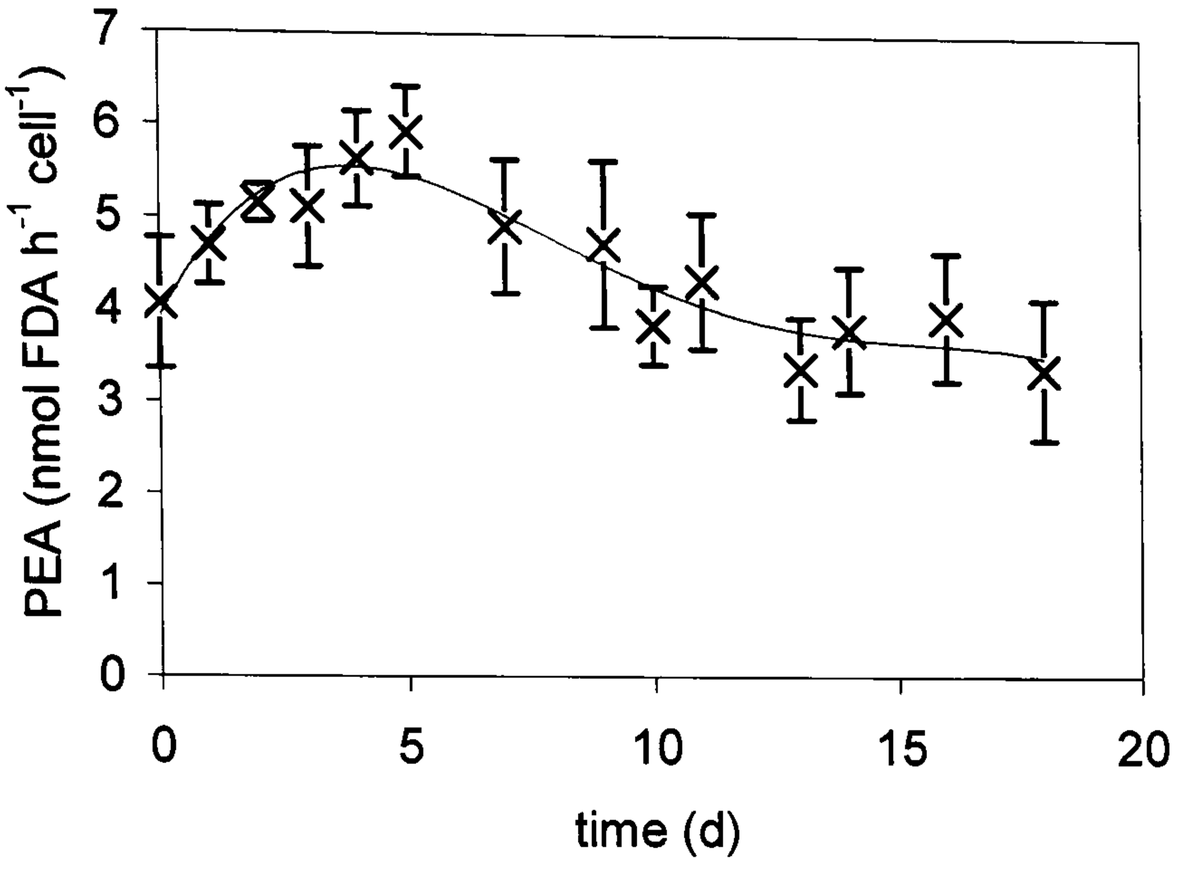


Figure IV-5. Particulate esterase activity per cell (nmol FDA h^{-1} L^{-1}) in cultures of *Skeletonema marinoi*. Data are means of replicates from 6 independent experiments. Error bars are standard deviations.

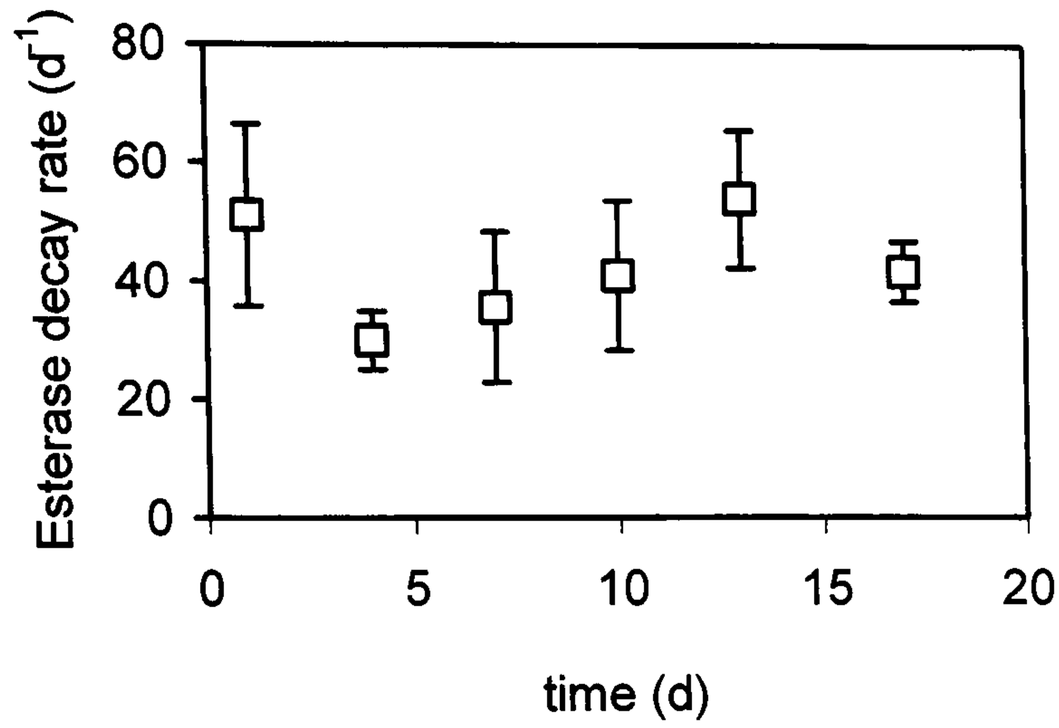


Figure IV-6. Esterase decay rate (d^{-1}) in cultures of *Skeletonema marinoi*. Data are means of replicates from 6 independent experiments. Error bars are standard deviations

Chl *a* concentrations increased during the exponential phase with a peak at 552 ± 7 µg L^{-1} and then decreased immediately in the stationary and declining phase of growth (Figure IV-7). The PEA Chl a^{-1} increased from 2.2 ± 0.3 to 10.3 ± 1.3 nmol FDA h^{-1} µg Chl a^{-1} in the exponential and stationary phases, and then decreased in the declining phase (Figure IV-7). The wide variability of PEA Chl a^{-1} during the growth cycle has already been shown with other phytoplankton species (Berman and Wynne 2005), and therefore the use of a fixed value of PEA Chl a^{-1} together with measured *in situ* Chl *a* concentrations to derive the PEA concentrations of ambient phytoplankton populations, as used by Agusti et al. (1998), would lead to inaccurate estimation of lysis rates. PEA will therefore be calculated by direct measurement of PEA in our *in situ* sampling series, assuming that PEAs from zooplankton and bacteria are negligible.

In summary, Table IV-1 shows the optimal practical protocol.

II.4. Conclusions

The results presented here show that esterase activity is a reliable quantitative tracer of phytoplankton cell lysis. In addition, a better estimation of the percentage of dead cells is obtained with this method, which is non-invasive and does not require cell counting. Although the lysis rate estimates may be biased by the presence of intracellular esterases in non-photosynthetic organisms, the method is attractive to study phytoplankton cell lysis at sea.

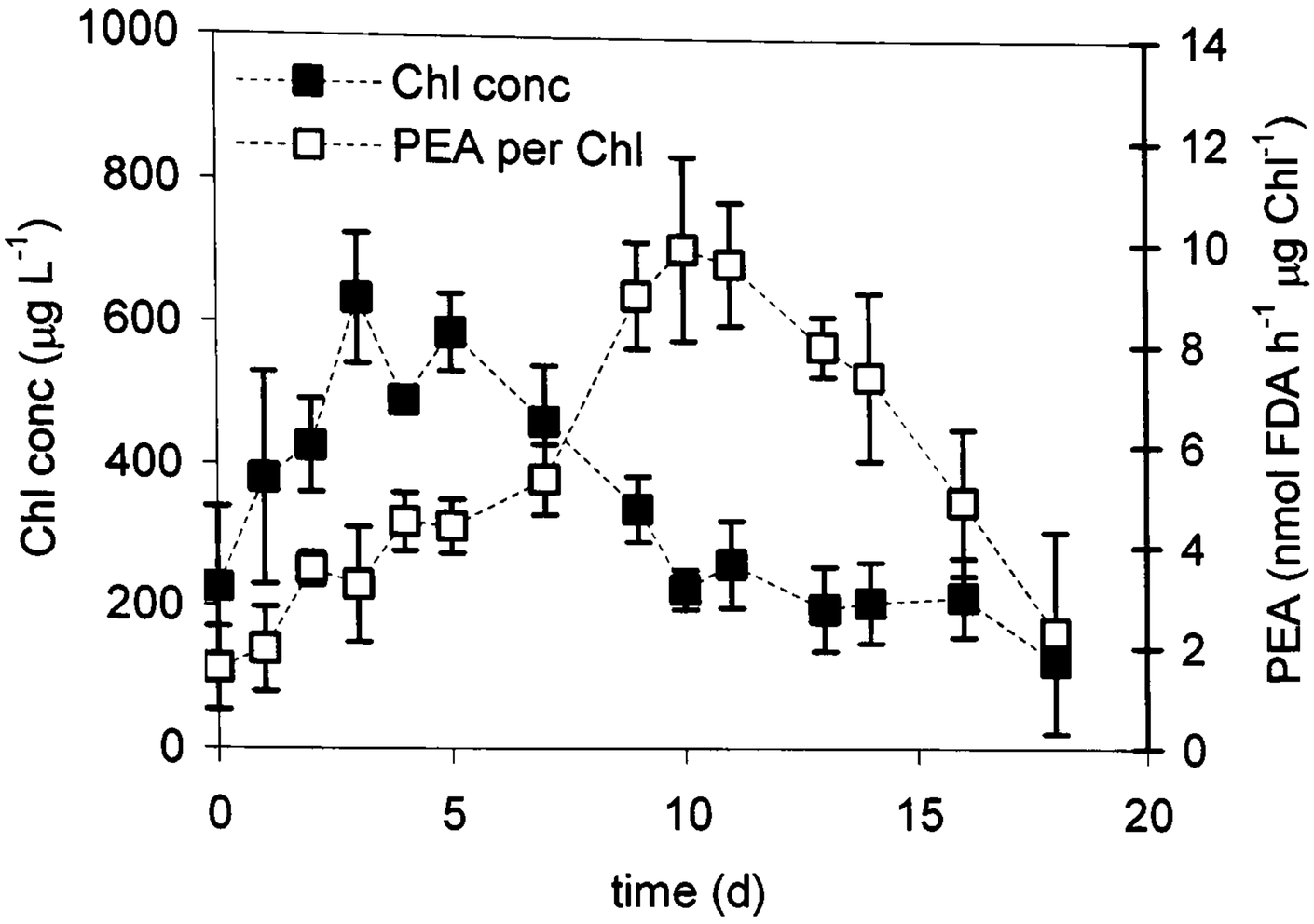


Figure IV-7. Chl *a* concentration (µg L^{-1}) and particulate esterase activity per chlorophyll *a* (nmol FDA h^{-1} µg Chl a^{-1}) in cultures of *Skeletonema marinoi*. Data are means of replicates from 6 independent experiments. Error bars are standard deviations

Table IV-1. Optimal practical protocol together with the equations to be used for the estimation of phytoplankton lysis rate.

<table>
<tr><th></th><th>Estimation of Total EA</th><th>Estimation of DEA</th><th>Estimation of NEH</th><th colspan="2">Estimation of T1/2</th></tr>
<tr><td rowspan="5">1</td><td rowspan="5">12 mL unfiltered sample</td><td rowspan="5">12 mL of sample gently filtered through 0.22 µm filter</td><td rowspan="5">12 mL of sample filtered through < 10kDa filter by centrifugation (4000g for 10 minutes)</td><td colspan="2">25 mL of sample gently filtered through 0.22 µm filter</td></tr>
<tr><td colspan="2">incubated at in situ water temperature in the dark for a 24-h period</td></tr>
<tr><td colspan="2">Split the sample in two equal parts :</td></tr>
<tr><td>12 mL already filtered through 0.22 µm filter</td><td>12mL filtered through < 10kDa filter by centrifugation (4000g for 10 minutes)</td></tr>
<tr><td></td><td colspan="4"></td></tr>
<tr><td>2</td><td colspan="4">Add into each 1.9 mL sample, in triplicates, 100 µL Lysis buffer (Tris-HCl pH 8.0, EDTA, final concentrations 500 µmol L^{-1}) and 20 µL FDA (in acetone; final concentration 20 µmol L^{-1})</td></tr>
<tr><td>3</td><td colspan="4">READ the fluorescence
(excitation at 450 nm and emission at 520 nm)</td></tr>
<tr><td>4</td><td colspan="4">1h-incubation at 20°C</td></tr>
<tr><td>5</td><td colspan="4">READ the fluorescence
(excitation at 450 nm and emission at 520 nm)</td></tr>
<tr><td>6</td><td colspan="4">Add 20 µL Fluorescein as internal standard
(final concentration 5 nmol L^{-1})
and READ the fluorescence again</td></tr>
</table>

III. Phytoplankton lysis rates in the Northern Adriatic Sea

(This study was conducted within the framework of an interdisciplinary collaborative project focused on the biological oceanography of the Northern Adriatic Sea (INTERREG Italy-Slovenia Project)

III.1. Introduction

In aquatic environments, losses of phytoplankton biomass occur through respiration, exudation of organic matter, grazing, sedimentation, and cell lysis. With the exception of cell lysis, these processes have been extensively studied in many coastal areas. Phytoplankton lysis may be caused by the action of algal viruses (for review Brussaard 2004 and Suttle 2005; Bidle et al. 2007)) and bacterial attack (Cole 1982; Mitsutani et al. 1992), by environmental stress, such as light and nutrient limitation (Brussaard et al. 1997, Berges and Falkowski 1998; Segovia et al. 2003) or endogenous processes, e .g. genetically-based cell death (Berman-Frank et al. 2004, Bidle and Falkowski 2004; Franklin et al. 2006). For example, lysis rates in *Ditylum brightwellii* in P-limited continuous cultures increased from 0.01 to 0.07 d^{-1} with decreasing growth rates (Brussaard et al. 1997).

Attempts to measure i*n situ* lysis rates by the esterase method were performed by Van Boekel et al. (1992) during a *Phaeocystis* spring bloom in Dutch coastal waters and showed that lysis rates, ranging from 0.01 - 0.25 d^{-1}, can play an important role in

the dynamics and fate of the bloom. More extensive studies in the same area indicated seasonality in the lysis rates of phytoplankton (Brussaard et al. 1995, 1996), with relatively low lysis rates in winter (< 0.1 d^{-1}), a peak at the end of the spring bloom (0.3 d^{-1}), and relatively high lysis rates in summer (0.05 - 0.3 d^{-1}) (Brussaard et al. 1996). A comparable pattern in the seasonality of phytoplankton lysis rates was found in the Northwestern Mediterranean Sea, with maximum values up to 1.5 d^{-1} in summer (Agusti and Duarte 2000). Such high rates of phytoplankton lysis seems intuitively unrealistic and may be overestimated due to methodological uncertainties detailed in section 1. Although the published quantitative results for phytoplankton lysis obtained by the esterase method have been subject to some debate (Agusti and Duarte 2002; Riegman et al. 2002), they have raised awareness of the potential importance of algal lysis as a process leading to the release of dissolved organic matter in aquatic ecosystems (Kirchman 1999).

It should be noted that cell lysis caused by osmotic stress can also trigger the production of toxic compounds, such as polyunsaturated aldehydes in diatoms (PUAs) (Wendel and Juttner 1996) and chrysophytes (Watson et al. 2001). Some preliminary results suggest that high lysis rates occur in final stages of PUA-producing *Skeletonema* blooms (Casotti et al. 2004) and therefore substantial amounts of PUAs may be released into seawater during this period. Since the PUAs are highly reactive compounds and may affect the growth of cultured phytoplankton species as well as bacteria (see Chap III-A and -B), it is reasonable to expect that these compounds may play a role in determining the composition and diversity of natural planktonic communities, as proposed for other toxic molecules (Hay 1996).

To support the hypothesis of a potential release of PUAs following cell lysis, phytoplankton lysis rates were measured in March 2002, 2003, 2005 -2006 in the Northern Adriatic Sea, where a recurrent late winter bloom of the PUA-producing diatom *Skeletonema marinoi* is observed (Miralto et al. 1999; Casotti et al. 2004), and in June 2003 and 2004 for comparison with respect to bloom periods. In parallel, potential cell PUA production was estimated during *Skeletonema* blooms. Preliminary data on the quantification of dissolved PUAs, performed in March 2005 and 2006, are also presented.

III.2. Material and Methods

III.2.1 Investigation area and sampling

Four oceanographic cruises were conducted in the Northern Adriatic Sea, three in March (2004-2005-2006) and one in June (2004). Additional data were obtained by Dr. R. Casotti during cruises in the same area conducted in February-March 2002 and June 2003. Samples were taken at the surface from stations located along three coast-to-offshore transects (Figure IV-8). In March 2005 and 2006, two different fixed stations were also sampled every 3 h for 27 h (Figure IV-8).

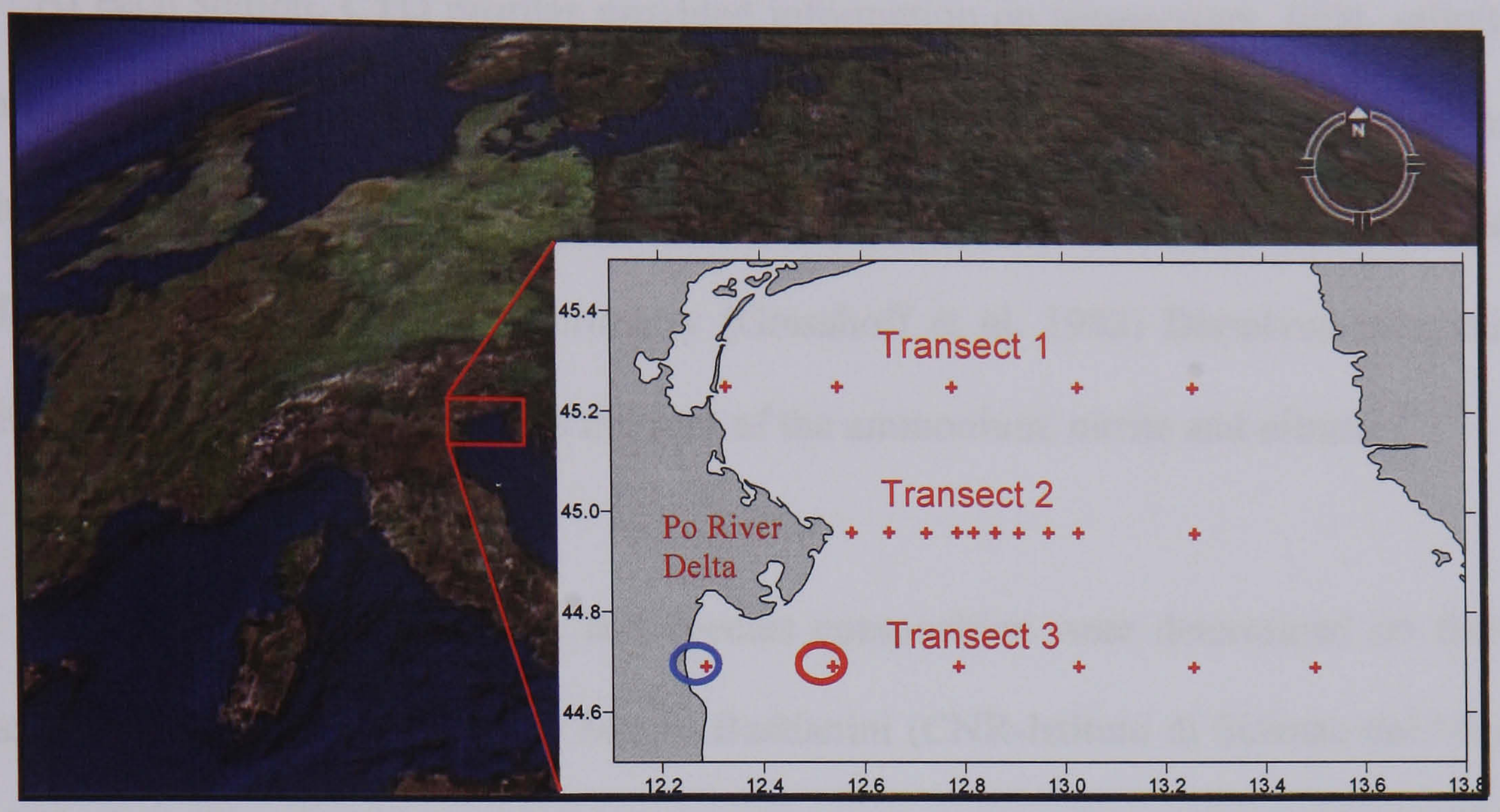


Figure IV-8. The research area with the sampling stations. The two stations indicated by blue and red circles were sampled over a 24 h-period in March 2005 and 2006, respectively.

At each station, CTD profiles provided information on temperature, light, salinity and *in situ* fluorescence data. Dissolved nutrients (phosphate, silicic acid, ammonium, nitrite and nitrate) were analyzed by Dr. Francesco Acri (CNR-Istituto di Scienze del Mare of Venice, IT) using colorimetry (Grasshoff et al. 1983) Dissolved inorganic nitrogen (DIN) was calculated as the sum of the ammonium, nitrite and nitrate.

Phytoplankton cell numbers and species composition were determined on fixed samples (formaldehyde 4 %) by Mauro Bastianini (CNR-Istituto di Scienze del Mare of Venice, IT) after concentration by sedimentation according to Utermöhl (1958), using an inverted microscope (Zeiss Axiovert 35), at a magnification of 400x.

Samples for particulate C and N (POC and PON, respectively) were filtered on acidified pre-combusted (450 °C, 24 h) GF/F filters and stored at -80 °C until analysis, using a CHN elemental analyzer (Perkin Elmer 2400). Analyses were performed by Dr. Daniele Cassin (CNR-Istituto di Scienze del Mare of Venice, IT).

Samples for Chl *a* analysis were obtained by gentle filtration of water samples over GF/F filters and stored at -80 °C until further analysis. The filters were ground in 100% methanol and the extract injected into a Beckman System Gold HPLC. Analysis of Chl *a* concentrations were performed by Dr. Christophe Brunet (Zoologica di Napoli, IT).

III.2.2 Lysis rates

Phytoplankton lysis rates were estimated following an improved version of the esterase method (Riegman et al. 2002) tested *in vivo* on the diatom *Skeletonema marinoi* Sarno & Zingone (see Table IV-1). Dissolved and particulate esterase activities were measured in three methodological replicates and the coefficient of variation averaged ~ 5%. The esterase decay rates were measured at each station.

III.2.3 Potential cell PUA production

In March 2002 and 2004, the quantification of potential cell PUA production was performed by Dr. G. Romano (Stazione Zoologica di Napoli) following the method of d'Ippolito et al. (2003), at 6 stations of transect 1. Cells were harvested by centrifugation (1200g, 10 min) and pellets (10^7 cells) were frozen until further analysis.

In March 2005 and 2006, the quantification of PUA production was performed following Wichard et al. (2005b) by Dr. Thomas Wichard (Max Planck Institute of Jena, DE) and Drs. Charles Vidoudez (Schiller University of Jena, DE) at 12 and 17 stations of the three transects. Briefly, 6-8 L of seawater were filtered on 1.2 µm GF/C filters. Cells were resuspended in 0.5 mL of filtered seawater by carefully pipetting. Then 1 mL of the derivatizing agent O-2,3,4,5,6-pentafluorobenzyl hydroxylamine hydrochloride (25 mmol L^{-1} PFBHA-HCl in 100 mmol L^{-1} Tris-HCl pH 7.0, Fluka)

was added together with 5 µL of internal standard (1 mmol L^{-1} Benzaldehyde in methanol) into the sample. Each sample was sonicated for 1 min (B. Braun Sonicator 1000L, GR) to disrupt cell integrity and to trigger PUA production Samples were then incubated for at least 30 min at room temperature and successively frozen in liquid nitrogen and stored at -80°C until further analysis. PUA production was calculated by dividing the PUA concentration by the number of *Skeletonema marinoi* cells.

III.2.4 Dissolved PUA

Detection and quantification of dissolved PUAs was performed following the method of Wichard et al. (in prep.) by Dr. Thomas Wichard (Max Planck Institute of Jena, DE) and Drs. Charles Vidoudez (Schiller University of Jena, DE) at 3 and 11 stations, respectively. One liter of surface seawater was gently filtered through 1-2 cm layer of dry commercial washed sea sand using a Büchner funnel with two layers of 25 µm Whatman filter paper. The filtered seawater, together with 5 µL of internal standard (1 mmol L^{-1} Benzaldehyde in methanol), was run through a C18 cartridge column (Chromabond C18 ec, Machery-Nagel) and the PUAs eluted by adding 6 ml of the derivatizing reagent (25 mmol L^{-1} PFBHA in methanol). The samples were incubated 1 h at room temperature, successively frozen in liquid nitrogen and stored at -80°C until further analysis.

III.2.5 Statistical analysis

The comparison of means was performed using the program Prism 4 (GraphPad Software, San Diego, USA). Principal Component Analysis (PCA) was used for dividing the data set into homogeneous groups. Once groups were chosen, another PCA was performed to determine whether lysis rate estimates were correlated with environmental variables of this group. The variables considered were silicic acid, dissolved inorganic nitrogen (as the sum of ammonium, nitrate and nitrite), phosphate, chlorophyll *a* concentrations, molar C:N ratio, total phytoplankton, diatom and *Skeletonema marinoi* concentrations. Sea surface temperature, irradiance and turbidity were excluded from the PCA because values showed very little variation among the stations (data not shown). PCA was performed using the program R (R Development Core Team, GNU General Public License, http://www.r-project.org/) with the help of Dr. Laurent Dubroca (Stazione Zoologica di Napoli, IT). The analysis was carried out on centered data (by subtracting the means from the individual values).

III.3. Results

III.3.1 Environmental variables

A trophic gradient, decreasing from West to East was always observed due to nutrient-rich Po river water spreading along the coastal area, especially visible along transects 2 and 3 (Figure IV-9, 10 and 11). The effect of the river-diluted water induced a strong enrichment in inorganic nutrients at the coastal stations, such as at the western station 1 of transect 2 in March 2004 where phosphate (Pi) and dissolved inorganic nitrogen (DIN) concentrations reached 362 and 342 μmol L^{-1} (Figure IV-10 and 11). Consequently, for both seasons, inorganic nutrient concentrations were lower in transect 1 (with 2.5 ± 1.7 μmol Si L^{-1}, 5.0 ± 4.5 μmol DIN L^{-1}, 0.08 ± 0.05 μmol Pi L^{-1}) than in transect 2 (with 6.0 ± 13.6 μmol Si L^{-1}, 19.7 ± 60.8 μmol DIN $L^{-1}$and 0.18 ± 0.44 μmol Pi L^{-1}) and 3 (with 4.4 ± 3.1 μmol Si L^{-1}, 18.4 ± 41.2 μmol DIN $L^{-1}$and 0.12 ± 0.21 μmol Pi L^{-1}) (Table IV-2). On average, the amount of inorganic nutrients were higher in March (with a mean value of 6.4 ± 4.0 μmol Si L^{-1}, 21.5 ± 16.6 μmol DIN L^{-1} and 0.19 ± 0.08 μmol Pi L^{-1}) than in June (with a mean value of 1.5 ± 2.1 μmol Si L^{-1}, 6.2 ± 4.5 μmol DIN L^{-1} and 0.07 ± 0.02 μmol Pi L^{-1}) (Table IV-2).

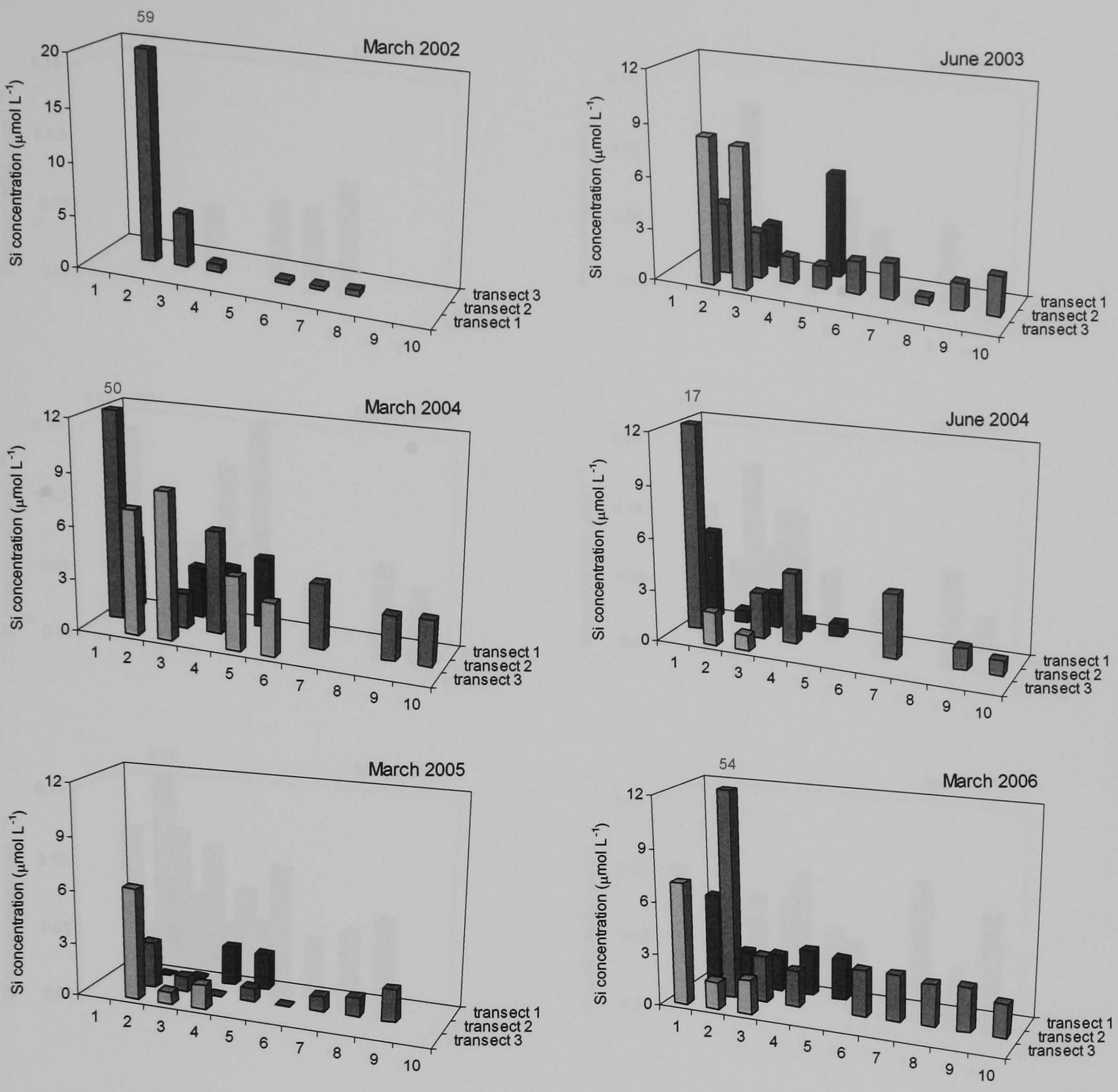


Figure IV-9. Silicic acid concentrations (µmol L^{-1}) for each station of the three transects sampled in March 2002, 2004-2006 and June 2003-2004 in the Northern Adriatic Sea.

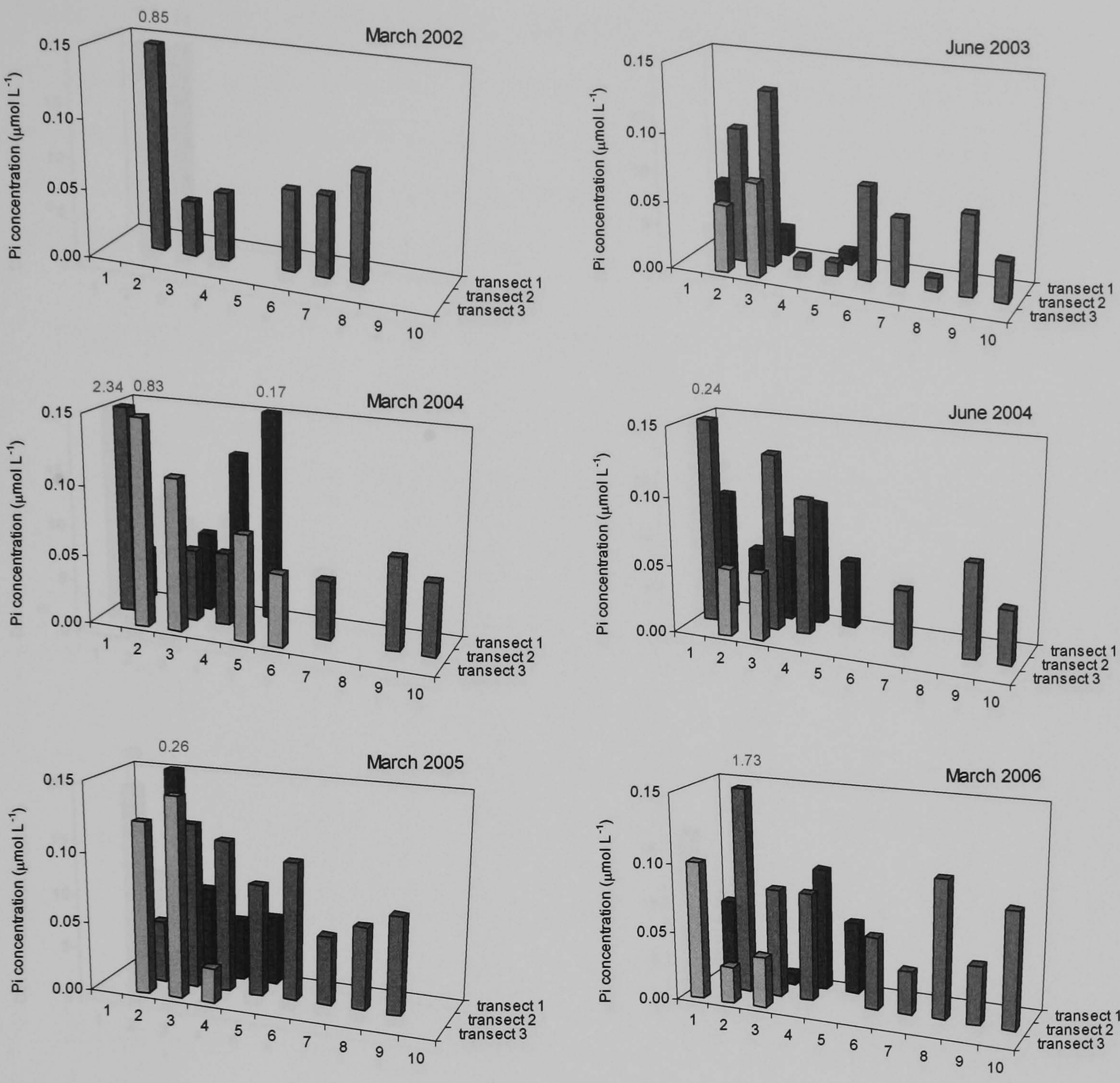


Figure IV-10. Phosphate concentrations (µmol L^{-1}) for each station of the three transects sampled in March 2002, 2004-2006 and June 2003-2004 in the Northern Adriatic Sea.

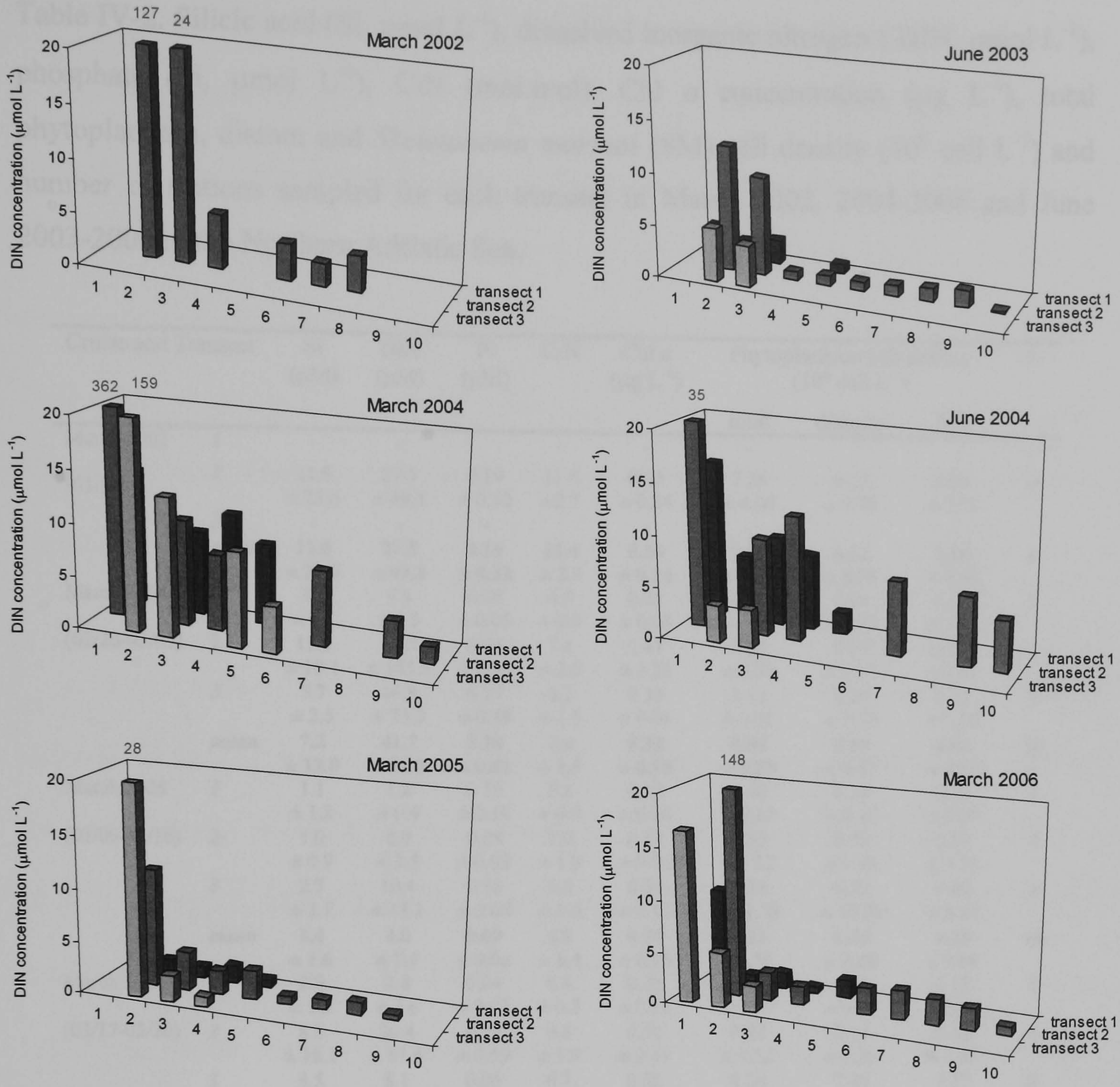


Figure IV-11. Dissolved inorganic nitrogen concentrations (µmol L^{-1}) for each station of the three transects sampled in March 2002, 2004-2006 and June 2003-2004 in the Northern Adriatic Sea.

Table IV-2. Silicic acid (Si, μmol L^{-1}), dissolved inorganic nitrogen (DIN, μmol L^{-1}), phosphate (Pi, μmol L^{-1}), C:N (mol:mol), Chl *a* concentration (μg L^{-1}), total phytoplankton, diatom and *Skeletonema marinoi* (SM) cell density (10^6 cell L^{-1}) and number of stations sampled for each transect in March 2002, 2004-2006 and June 2003-2004 in the Northern Adriatic Sea.

Cruise and Transect		Si (μM)	DIN (μM)	Pi (μM)	C:N	Chl a (μg L^{-1})	Phytoplankton cell density (10^6 cell L^{-1})			N
							total	diatoms	*SM*	
March 2002	*1*	-	-		-	-	-			-
	2	11.0 ± 23.6	27.5 ± 49.3	0.19 ± 0.32	11.4 ±2.7	0.26 ± 0.24	7.25 ± 4.06	6.12 ± 3.76	4.06 ± 2.72	6
(03/12)	*3*	-	-	-		-	-	-	-	-
	mean	**11.0 ± 23.6**	**27.5 ± 49.3**	**0.19 ± 0.32**	**11.4 ± 2.7**	**0.26 ± 0.24**	**7.25 ± 4.06**	**6.12 ± 3.76**	**4.06 ± 2.72**	**6**
March 2004	*1*	3.7 ± 0.7	9.6 ± 2.3	0.08 ± 0.05	6.9 ± 0.9	0.21 ± 0.04	0.74 ± 0.21	0.04 ± 0.03	0.00 ± 0.01	5
(02/26-03/03)	*2*	11.6 ± 19.1	65.3 ± 145.5	0.43 ± 0.93	7.4 ± 2.0	0.41 ± 0.25	0.83 ± 0.39	0.09 ± 0.17	0.01 ± 0.01	6
	3	5.7 ± 2.5	46.8 ± 75.3	0.27 ± 0.38	6.2 ± 1.5	0.33 ± 0.04	1.11 ± 0.31	0.29 ± 0.30	0.22 ± 0.28	4
	mean	**7.2 ± 12.0**	**41.7 ± 96.9**	**0.28 ± 0.61**	**7.0 ± 1.5**	**0.33 ± 0.18**	**0.84 ± 0.33**	**0.10 ± 0.17**	**0.04 ± 0.11**	**16**
March 2005	*1*	1.1 ± 1.2	1.2 ± 0.4	0.10 ± 0.10	6.8 ± 0.9	0.13 ± 0.04	0.46 ± 0.13	0.14 ± 0.10	0.08 ± 0.06	4
(03/09-03/16)	*2*	1.0 ± 0.9	2.9 ± 3.5	0.08 ± 0.03	7.0 ± 1.8	0.17 ± 0.06	1.30 ± 1.53	0.75 ± 0.96	0.30 ± 0.21	8
	3	2.7 ± 3.1	10.4 ± 15.1	0.10 ± 0.06	6.0 ± 1.0	0.36 ± 0.11	7.58 ± 11.38	6.53 ± 10.08	5.40 ± 8.29	3
	mean	**1.4 ± 1.6**	**4.0 ± 7.1**	**0.09 ± 0.06**	**6.8 ± 1.4**	**0.20 ± 0.10**	**2.33 ±5.21**	**1.74 ± 4.60**	**1.26 ± 3.80**	**15**
March 2006	*1*	2.9 ± 1.3	2.8 ± 3.6	0.04 ± 0.03	6.8 ± 0.8	0.26 ± 0.11	0.39 ± 0.37	0.14 ± 0.30	0.13 ± 0.28	5
(03/17-03/20)	*2*	8.8 ± 18.1	20.4 ± 51.6	0.27 ± 0.59	9.8 ± 1.9	0.58 ± 0.47	0.42 ± 0.32	0.14 ± 0.36	0.13 ± 0.35	8
	3	3.5 ± 3.0	8.1 ± 7.4	0.06 ± 0.04	6.2 ± 1.1	0.58 ± 0.63	8.28 ± 13.39	7.45 ± 12.56	7.27 ± 12.31	4
	mean	**6.0 ± 12.8**	**12.6 ± 36.3**	**0.16 ± 0.42**	**8.2 ± 2.2**	**0.50 ± 0.45**	**1.88 ± 5.84**	**1.51 ± 5.46**	**1.47 ± 5.34**	**17**
June 2003	*1*	2.6 ± 2.5	2.0 ± 0.8	0.03 ± 0.02	8.0 ± 0.5	0.27 ± 0.40	1.33 ± 1.68	0.56 ± 0.98	0.00 ± 0.01	4
(05/29-06/08)	*2*	2.0 ± 1.0	3.2 ± 4.4	0.05 ± 0.04	6.4 ± 1.0	0.32 ± 0.40	1.35 ± 1.02	1.19 ± 2.02	0.02 ± 0.03	9
	3	8.3 ± 0.2	4.5 ± 0.8	0.06 ± 0.01	5.5 ± 0.0	0.34 ± 0.27	0.71 ± 0.32	0.25 ± 0.14	0.00 ± 0.00	2
	mean	**3.0 ± 2.6**	**3.0 ± 3.5**	**0.05 ± 0.04**	**6.7 ± 1.2**	**0.31 ± 0.36**	**1.26 ± 1.12**	**0.90 ± 1.64**	**0.01 ± 0.02**	**15**
June 2004	*1*	1.9 ± 2.0	8.0 ± 4.8	0.07 ± 0.02	8.0 ± 0.7	1.38 ± 0.60	11.37 ± 6.30	8.10 ± 5.22	0.02 ± 0.04	5
(05/28-06/10)	*2*	4.9 ± 5.9	12.5 ± 11.2	0.10 ± 0.07	6.9 ± 0.7	2.48 ± 1.84	20.65 ± 13.27	14.60 ± 12.83	0.05 ± 0.06	6
	3	1.4 ± 0.8	3.6 ± 0.0	0.05 ± 0.00	7.7 ± 1.9	1.56 ± 0.35	9.11 ± 1.57	6.42 ± 1.60	0.00 ± 0.00	2
	mean	**0.08 ± 0.05**	**9.4 ± 8.4**	**0.08 ± 0.05**	**7.5 ± 1.0**	**1.92 ± 0.36**	**14.33 ± 9.73**	**10.16 ± 8.56**	**0.20 ± 0.42**	**13**

Chl *a* concentrations were highly variable, ranging from 0.05 to 0.93 μg L^{-1} in March and from 0.06 to 5.31 μg L^{-1} in June without a clear gradient (Figure IV-12). Average values were not significantly different between the different March cruises (0.34 ± 0.31 μg L^{-1}) (ANOVA, $p > 0.05$) while very high values of Chl *a* concentrations were observed in June 2004 (1.92 ± 0.36 μg L^{-1}) with respect to June 2003 (0.31 ± 36 μg L^{-1}) (Student *t*-test, $p < 0.001$) (Table IV-2). No significant differences were observed between the different transects (Tukey Multiple Comparion post-test, $p > 0.5$).

Average molar C:N ratios in March (7.83 ± 2.37 SD) were similar to those obtained in June (7.05 ± 1.16 SD) (ANOVA, p>0.1) (Table IV-2) and were close to the Redfield ratio (6.62). A gradient of the C:N ratio was observed in March 2002 and 2006 at transect 2, with higher values at the Eastern stations (11.55 ± 1.99 SD) where lower DIN concentrations were observed (2.71 ± 1.19 μmol L^{-1} SD) (Figure IV-11and 13). These results suggest that phytoplankton cells, mostly composed of *Skeletonema marinoi*, were experiencing nitrogen limitation at these stations.

In March, the phytoplankton was dominated by diatoms whose concentrations were very high in March 2002, 2005 and 2006, due to a bloom of *Skeletonema marinoi* at the Western stations enriched by the Po River (Table IV-2 and Figure IV-14). Cell concentrations reached 1.1 x 10^7 cell L^{-1} in March 2002, 1.8 x 10^7 cell L^{-1} in March 2005 and 2.2 x 10^7 cell L^{-1} in March 2006 (Figure IV-14), and represented on average 97% of the total phytoplankton in terms of cell numbers.

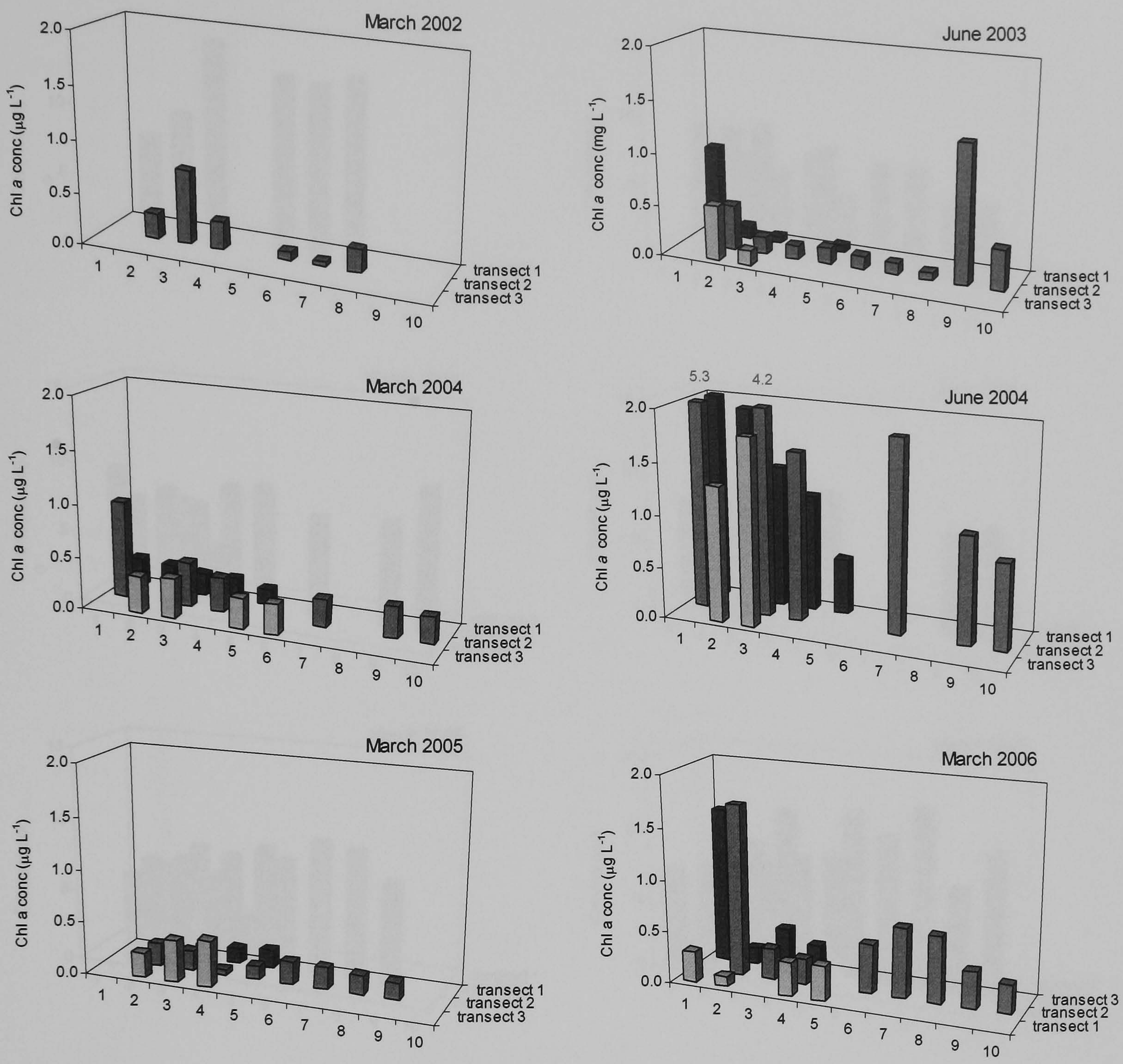


Figure IV-12. Chlorophyll *a* concentrations (µg L^{-1}) for each station of the three transects sampled in March 2002, 2004-2006 and June 2003-2004 in the Northern Adriatic Sea.

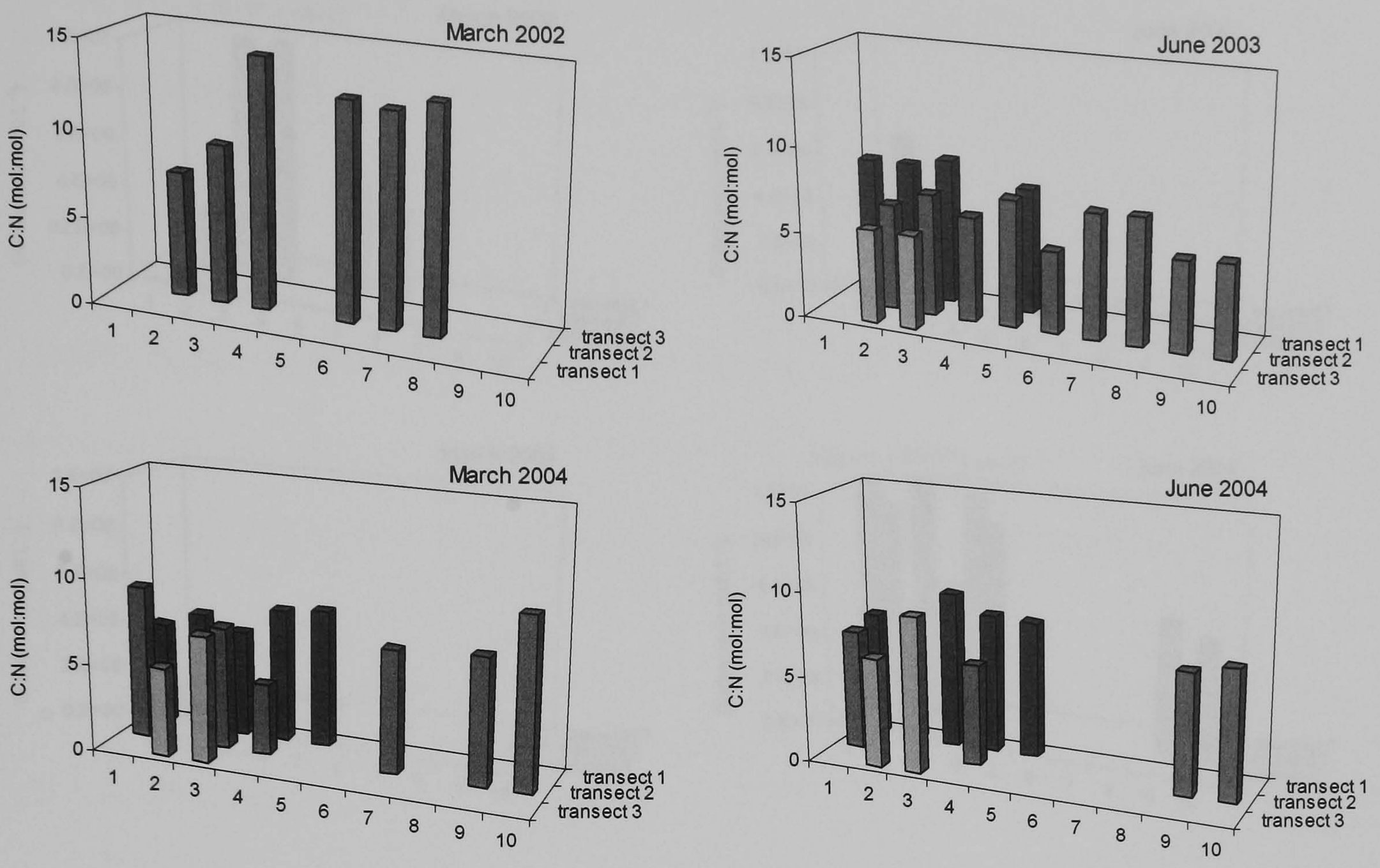


Figure IV-13. Molar C:N ratio (mol:mol) for each station of the three transects sampled in March 2002, 2004-2006 and June 2003-2004 in the Northern Adriatic Sea.

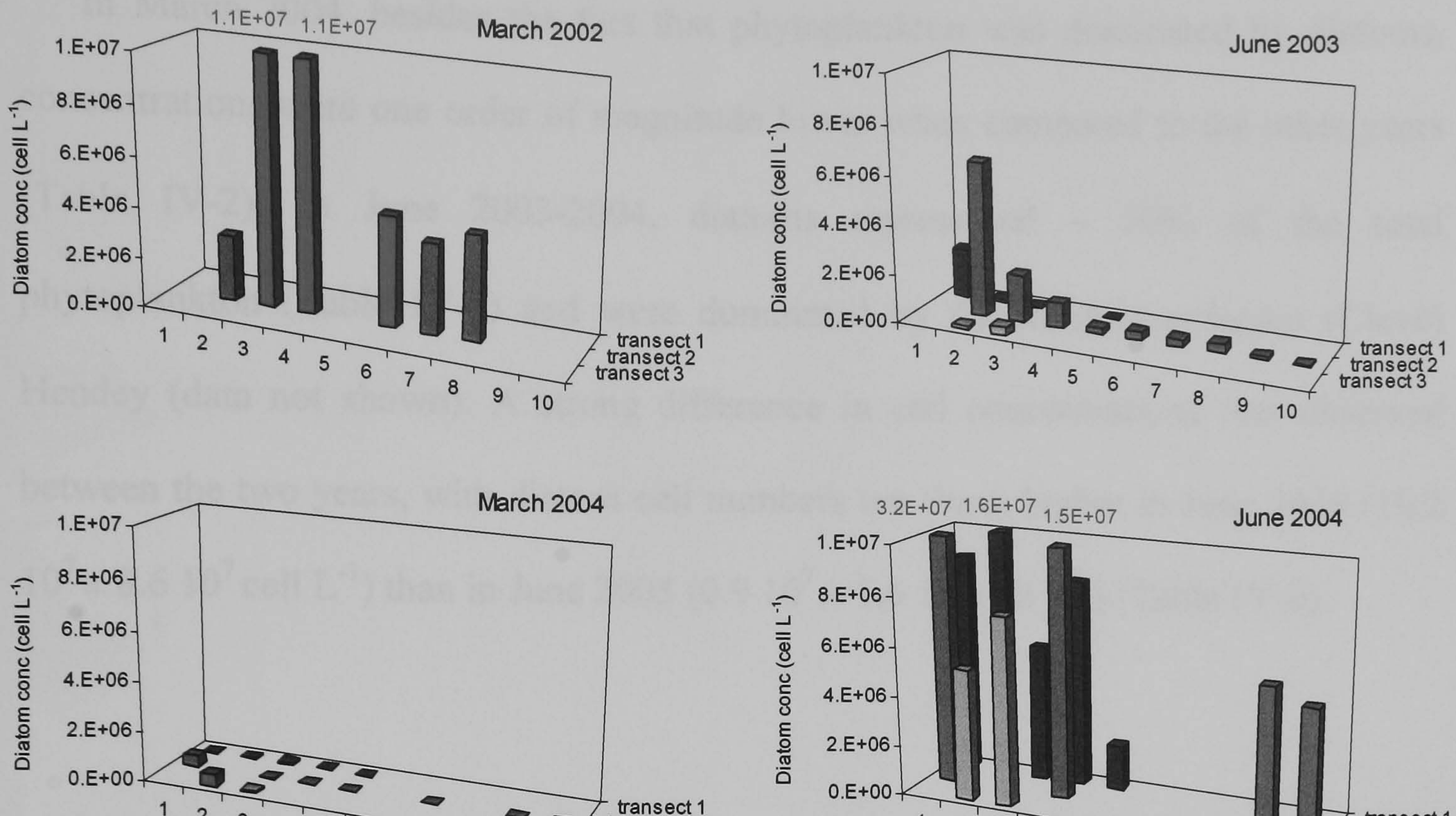


Figure IV-14. Diatom concentrations (cell L^{-1}) for each station of the three transects sampled in March 2002, 2004-2006 and June 2003-2004 in the Northern Adriatic Sea.

In March 2004, besides the fact that phytoplankton was dominated by diatoms, concentrations were one order of magnitude lower when compared to the other years (Table IV-2). In June 2003-2004, diatoms represented ~ 70% of the total phytoplankton (Table IV-2) and were dominated by *Cerataulina pelagica* (Cleve) Hendey (data not shown). A strong difference in cell concentrations was observed between the two years, with diatom cell numbers ten times higher in June 2004 ($10.2\ 10^7 \pm 8.6\ 10^7$ cell L^{-1}) than in June 2003 ($0.9\ 10^7 \pm 1.6\ 10^7$ cell L^{-1}) (Table IV-2).

III.3.2 Lysis rates

Lysis rates ranged in March from 0.03 to 0.38 d^{-1} with an average value of 0.16 ± 0.01 d^{-1}) (Figure IV-15). No significant differences were observed among the four late-winter cruises (ANOVA, $p > 0.05$) (Table IV-3). In June, lysis rates ranged from 0.02 to 0.25 d^{-1} with an average value of 0.09 ± 0.01 d^{-1} and were not significantly different between the two early summer cruises (Student's *t*-test, $p > 0.05$) (Figure IV-15and Table IV-3). The higher lysis rates in March as compared to June were highly significant (Tukey Multiple Comparison post-test, $p < 0.001$). In both seasons, transect 1 showed lower lysis rate values (0.08 ± 0.04 d^{-1}) than those obtained in transect 2 (0.16 ± 0.08 d^{-1}) and transect 3 (0.15 ± 0.06 d^{-1}) (Table IV-3).

Value of lysis rates presented here were calculated with equation 1 (linear model) and were on average 0.01% lower than those calculated with equation 4 (exponential model) (Student t-test, $p < 0.01$).

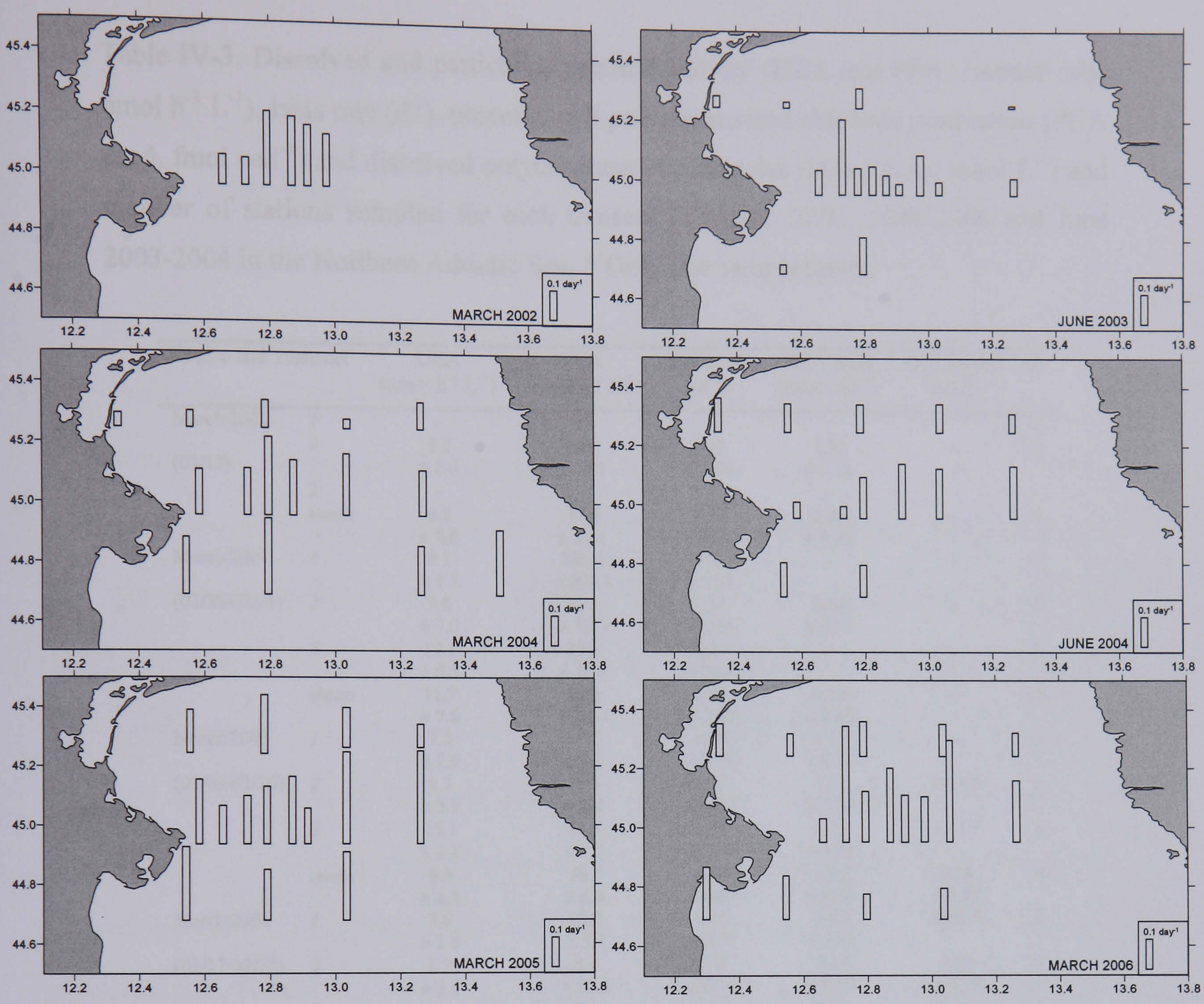


Figure IV-15. Lysis rate (d^{-1}) distribution in the Northern Adriatic sea in March 2002, 2004-2006 and June 2003-2004.

Table IV-3. Dissolved and particulate esterase activity (DEA and PEA, respectively, nmol h^{-1} L^{-1}), lysis rate (d^{-1}), potential cell polyunsaturated aldehyde production (PUA prod, fmol $cell^{-1}$) and dissolved polyunsaturated aldehydes (Diss PUAs, nmol L^{-1}) and number of stations sampled for each transect in March 2002, 2004-2006 and June 2003-2004 in the Northern Adriatic Sea. * Only one sample taken.

Cruise and Transect		DEA (nmol h^{-1} L^{-1})	PEA (nmol h^{-1} L^{-1})	Lysis rate (d^{-1})	PUA prod (fmol $cell^{-1}$)	Diss PUAs (nM)	N
March 2002	*1*	-	-	-	-	-	-
	2	5.2 ± 2.0	13.0 ± 10.1	0.17 ± 0.06	2.00 ± 1.22	-	6
(03/12)	*3*	-	-	-	-	-	-
	mean	**5.2 ± 2.0**	**13.0 ± 10.1**	**0.17 ± 0.06**	**2.00 ± 1.22**	-	**6**
March 2004	*1*	8.1 ± 9.7	260.4 ± 292.1	0.03 ± 0.03	-	-	5
(02/26-03/03)	*2*	9.8 ± 7.0	18.3 ± 15.3	0.17 ± 0.04	0.64 ± 0.68	-	6
	3	19.1 ± 9.5	27.7 ± 10.2	0.20 ± 0.04	-	-	4
	mean	**11.7 ± 7.8**	**30.3 ± 21.4**	**0.14 ± 0.07**	**0.64 ± 0.68**	-	**15**
March 2005	*1*	7.2 ± 2.6	18.2 ± 4.9	0.14 ± 0.05	1.10 ± 0.77	-	4
(03/09-03/16)	*2*	9.3 ± 3.9	16.7 ± 7.3	0.21 ± 0.07	1.11 ± 1.01	0.14 *	8
	3	15.1 ± 2.8	24.7 ± 5.2	0.21 ± 0.04	1.25 ± 1.08	0.17 *	3
	mean	**9.9 ± 4.3**	**18.7 ± 6.8**	**0.19 ± 0.07**	**1.41 ± 0.87**	**0.16 ± 0.02**	**15**
March 2006	*1*	3.0 ± 1.8	10.0 ± 5.4	0.10 ± 0.02	3.58 ± 4.81	0.60 *	5
(03/17-03/20)	*2*	5.7 ± 5.4	8.4 ± 5.33	0.21 ± 0.10	0.43 ± 0.38	1.09 ± 0.99	8
	3	3.1 ± 2.6	7.4 ± 4.2	0.13 ± 0.04	0.31 ± 0.57	0.17 ± 0.05	4
	mean	**4.3 ± 4.1**	**8.6 ± 4.9**	**0.16 ± 0.09**	**1.33 ± 2.86**	**0.79 ± 0.87**	**17**
June 2003	*1*	20.1 ± 1.7	2.6 ± 2.5	0.03 ± 0.03	-	-	4
(05/29-06/08)	*2*	21.2 ± 0.9	2.0 ± 1.0	0.09 ± 0.06	-	-	9
	3	22.0 ± 0.8	8.3 ± 0.2	0.08 ± 0.06	-	-	2
	mean	**16.0 ± 13.5**	**260.2 ± 164.0**	**0.07 ± 0.06**	-	-	**15**
June 2004	*1*	18.2 ± 0.2	1.9 ± 2.0	0.09 ± 0.02	-	-	5
(05/28-06/10)	*2*	19.7 ± 0.4	4.9 ± 5.9	0.13 ± 0.06	-	-	6
	3	19.3 ± 0.1	1.4 ± 0.8	0.11 ± 0.01	-	-	2
	mean	**30.8 ± 20.0**	**267.8 ± 133.7**	**0.11 ± 0.04**	-	-	**13**

For March 2002 and 2003, samples were taken by Dr. Raffaella Casotti (Stazione Zoologica di Napoli).

PCA performed on the entire data set discriminated three distinct groups: (1) March 2002, 2004-2006; (2) June 2003; (3) June 2004 (data not shown). Each group was analyzed independently. In the first group (March 2002, 2004-2006), variables were associated as following: chlorophyll *a* with inorganic nutrients (silicic acid, phosphate and dissolved inorganic nitrogen); diatoms with *Skeletonema* concentrations (as expected since the diatom population was dominated by *Skeletonema marinoi*); and lysis rates with molar C:N ratios (Table IV-4). Lysis rates and molar C:N ratios accounted for only 11% of the total variance, which indicated that these two variables were only slightly related. In the second group (June 2003), an association between diatoms, phosphate and dissolved inorganic nitrogen concentrations was found, as well as between silicic acid concentrations and molar C:N ratios. Lysis rates were not associated with any of the variables (Table IV-4). In the third group (June 2004), inorganic nutrient, total phytoplankton, diatom and Chl *a* concentrations were associated with each other and accounted for 72% of the total variance (Table IV-4). The total phytoplankton and diatom concentrations were positively correlated with inorganic nutrient concentrations (data not shown). Lysis rates and molar C:N ratios were also associated with one another and accounted for 21% of the total variance (Table IV-4). So, here again, lysis rates were only slightly related with C:N ratios.

To summarize, lysis rates was slightly associated with molar C:N ratios in March (2002, 2004-2006) and June 2004, but not with inorganic nutrient, Chl *a* or phytoplankton concentrations. So the environmental factors responsible for cell lysis remain poorly known.

Table IV-4. Summary of PCA using lysis rate, silicic acid (Si), dissolved inorganic nitrogen (DIN), phosphate (Pi), chlorophyll *a* concentrations (Chl *a*), molar C:N ratios, total phytoplankton, diatom and *Skeletonema marinoi* (SM) concentrations as variables and performed on three distinct groups: (1) March 2002, 2004-2006; (2) June 2003; (3) June 2004, in order to determine whether lysis rates are related to environmental variables.

Groups	Principal component (PC)	total variance	Identity of the major components
March 2002 2004 2005 2006	PC1	37%	Si, Pi, DIN and Chl *a* concentrations
	PC2	31%	total phytoplankton, diatoms and *SM* concentrations
	PC3	11%	Lysis rates and C:N ratios
June 2003	PC1	43%	Pi, DIN and diatom concentrations
	PC2	18%	Si concentrations and molar C:N ratios
	PC3	15%	Chl *a* concentrations
June 2004	PC1	72%	Si, Pi, DIN, Chl *a*, total phytoplankton and diatom concentrations
	PC2	16%	Lysis rates and molar C:N ratios
	PC3	5%	Lysis rates and molar C:N ratios

III.3.3 Potential PUA production and dissolved PUAs

Potential PUA production ranged from 0.00 to 10.55 fmol $cell^{-1}$ from March 2002-2006 with an average value of 1.27 fmol $cell^{-1}$ (± 1.96 fmol $cell^{-1}$ SD) (Figure IV-16). PUA production in March 2002 (2.00 ± 1.22 fmol $cell^{-1}$ SD), 2005 (1.41 ± 0.87 fmol $cell^{-1}$ SD) and 2006 (1.33 ± 2.86 fmol $cell^{-1}$ SD) were not significantly different (Tukey Multiple Comparison post-test, $p > 0.1$) while lower production was observed in March 2004 (0.64 ± 0.68 fmol $cell^{-1}$ SD) (Tukey Multiple Comparison post-test, $p < 0.05$) (Table IV-3). A positive correlation was found between PUA production and *Skeletonema* cell concentrations (Figure IV-17a, red dashes). The relationship between PUA production and lysis followed this pattern: it increased with increasing lysis rates according to a sigmoidal model and then dropped at the highest values of lysis rates (Figure IV-17b, trend indicated in red). Here again, highest PUA production was observed when lysis rates remained relatively low (Figure IV-17b, red crosses).

The quantification of dissolved PUAs in seawater was performed at two stations in March 2005 and more extensively in March 2006, with 17 stations sampled (Table IV-3 and Figure IV-18). Values ranged from 0.06 to 2.53 nmol L^{-1} with an average value of 0.79 ± 0.87 nmol L^{-1} in March 2006 (Table IV-3).

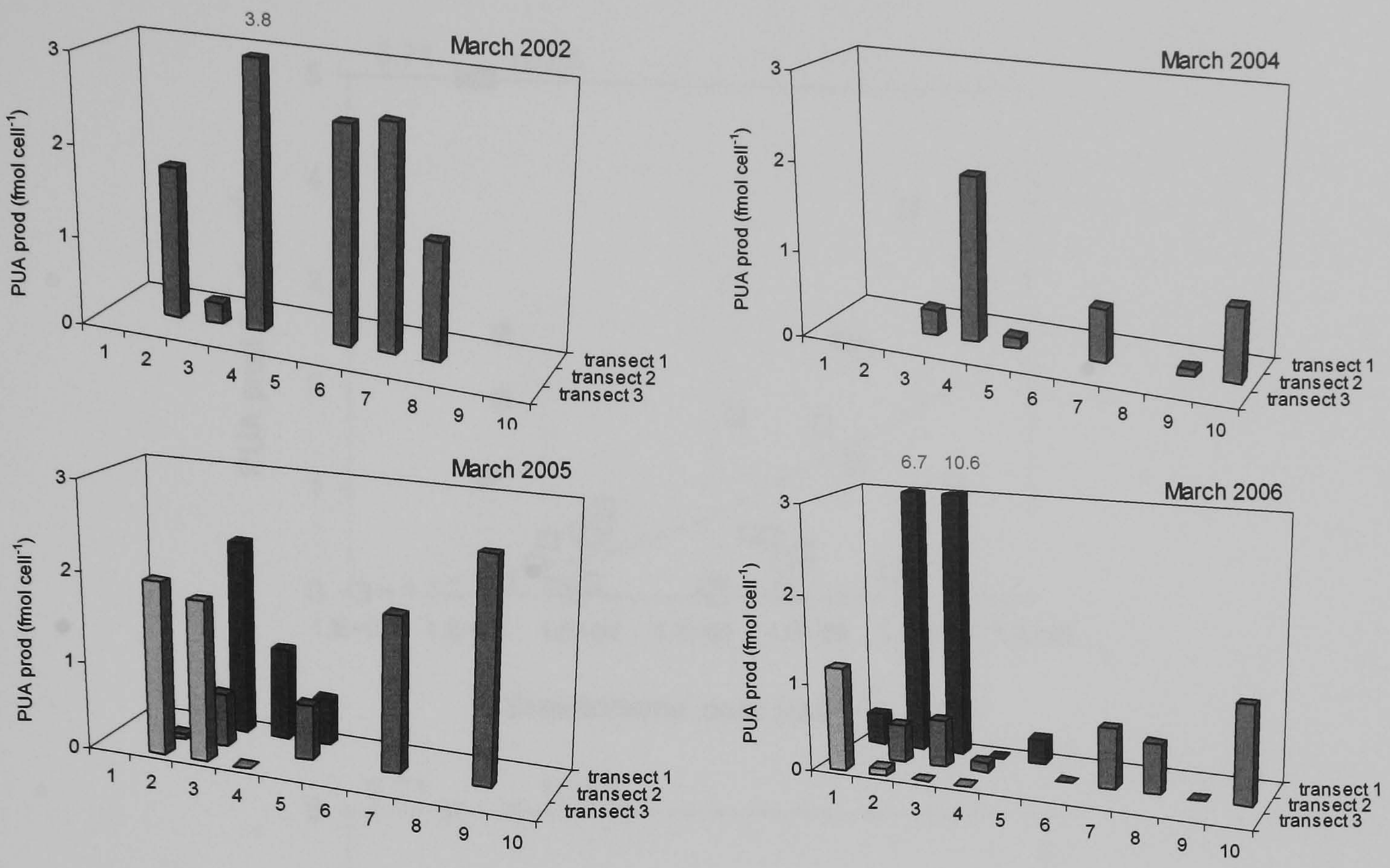
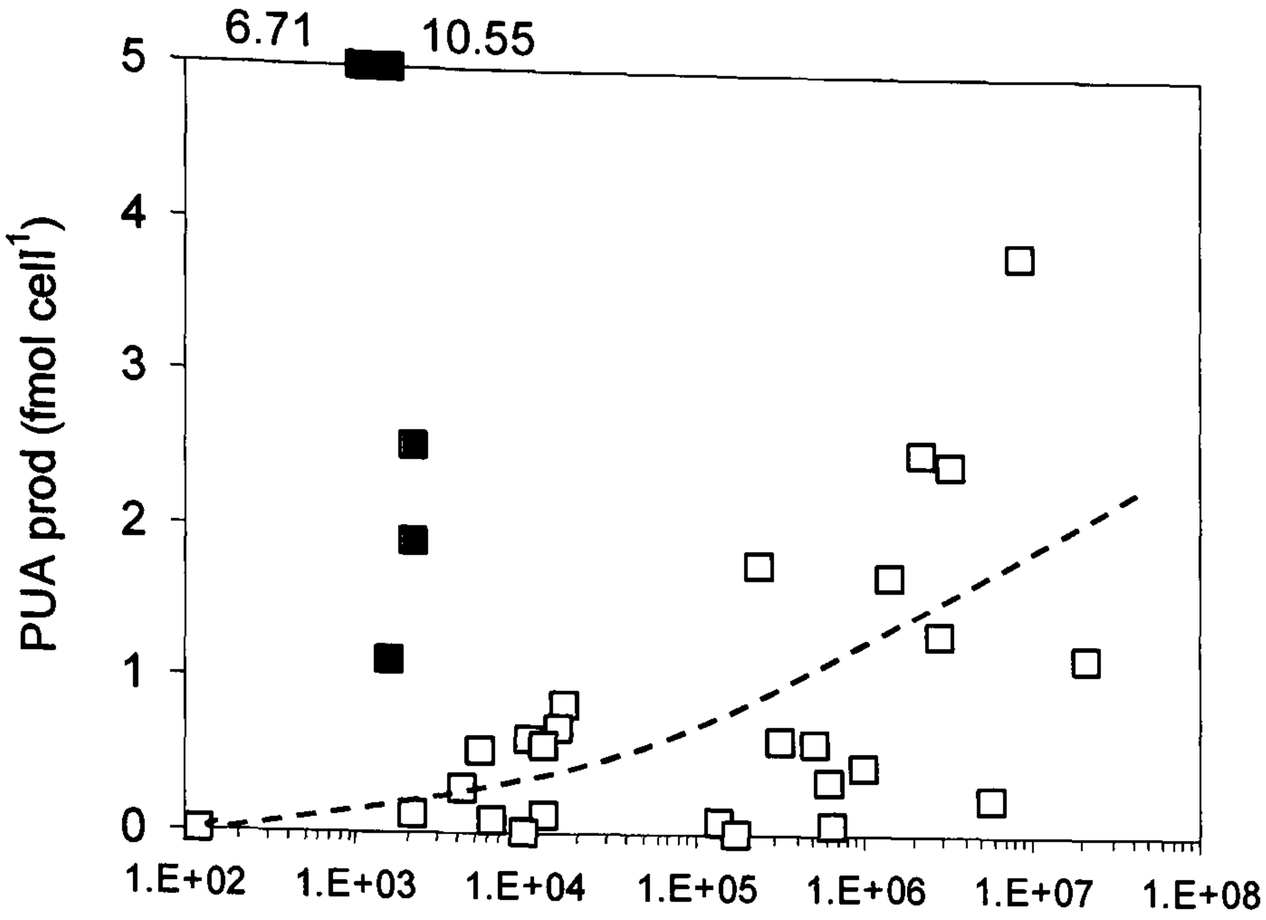


Figure IV-16. Potential polyunsaturated aldehyde production (fmol cell^{-1}) for each station of the three transects sampled in March 2002, 2004-2006 in the Northern Adriatic Sea.



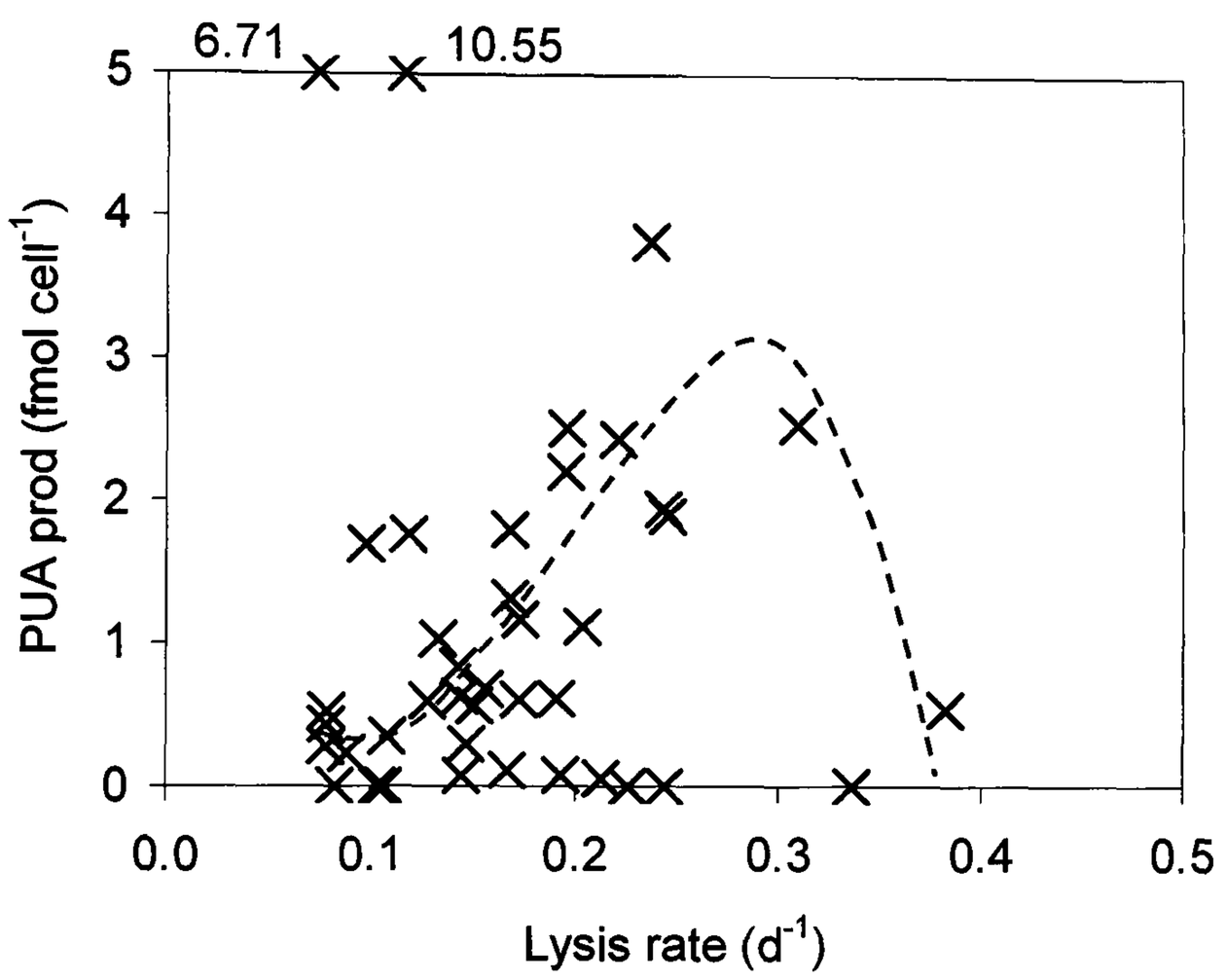


Figure IV-17. a) Relation between potential cell PUA production (fmol cell^{-1}) and *Skeletonema* concentration (cell L^{-1}). Red squares have been excluded form the regression curve (red dashes). b) Relation between potential cell PUA production (fmol cell^{-1}) and lysis rates (d^{-1}). Red crosses have been excluded form the regression curve (red dashes).

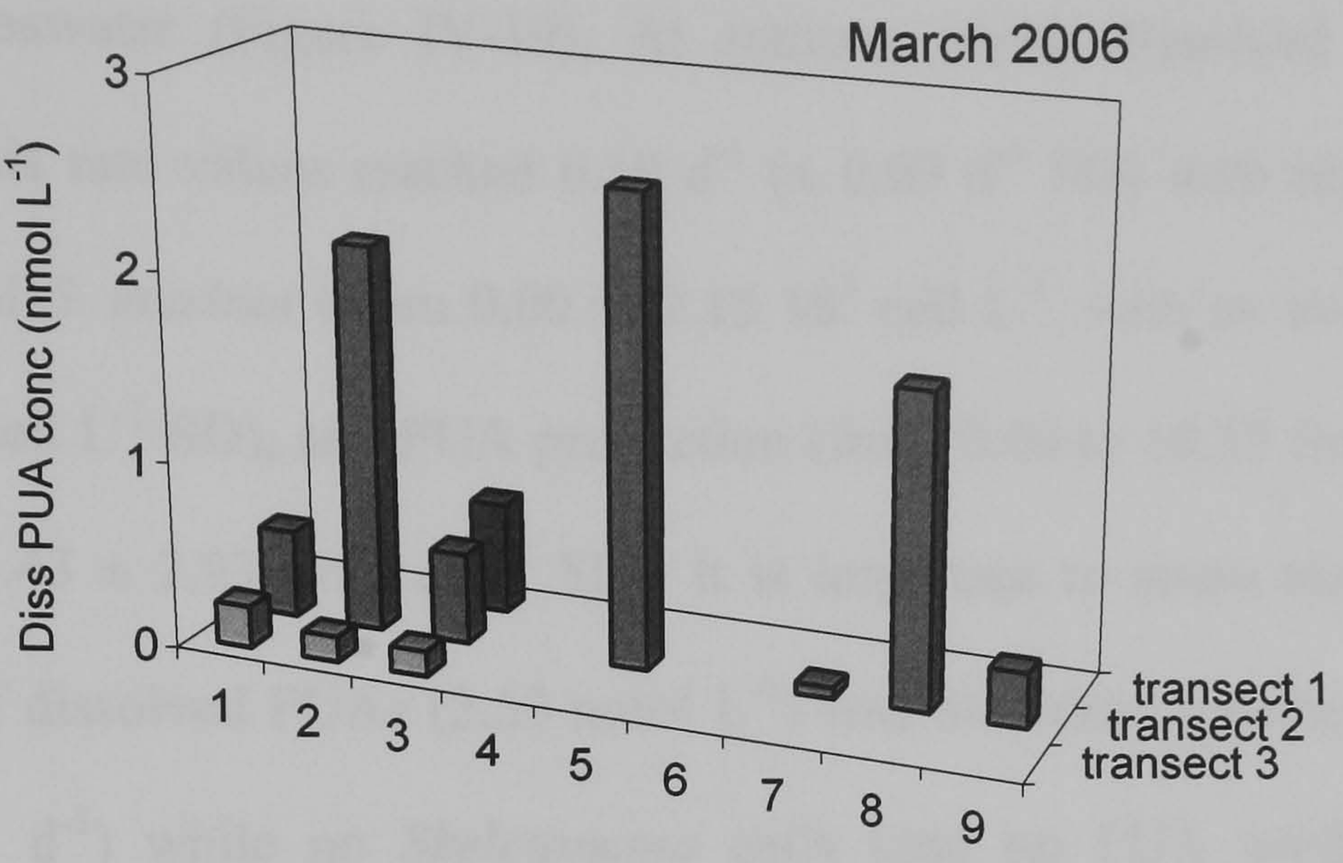


Figure IV-18. Dissolved polyunsaturated aldehyde concentrations (nmol L^{-1}) for each station of the three transects sampled in March 2006 in the Northern Adriatic Sea.

A good correlation was observed between lysis rates and the amount of dissolved PUAs in the seawater (Figure IV-19). At stations where dissolved PUAs were detected, the lysis rate values reached 0.19 d^{-1} (± 0.09 d^{-1} SD) with highly variable concentrations of *S. marinoi* (from 0.00 to 2.15 10^7 cell L^{-1}, with an average of 3.18 10^6 ± 7.17 10^6 cell L^{-1} SD), and PUA production (from 0.00 to 10.55 fmol $cell^{-1}$ with an average of 1.43 ± 2.93 fmol $cell^{-1}$ SD). It is important to stress that the highest concentration of dissolved PUAs (2.53 nmol L^{-1}) was measured together with a high lysis rate (0.24 d^{-1}) while no *Skeletonema* cells (and no PUA production) were observed.

24 h-time course experiments in March 2005 and 2006 were performed at two fixed stations where *Skeletonema* cells concentration represented 98% and 97% of the total phytoplankton cell numbers, with concentrations of 1.8 x 10^7 cell L^{-1} and 2.2 x 10^7 cell L^{-1}, respectively. During the 24 h-period, lysis rates ranged from 0.04 to 0.19 d^{-1} in March 2005 with average values at night of 0.13 ± 0.05 d^{-1} and 0.08 ± 0.05 d^{-1} during the light period (Figure IV-20a). Despite relatively few data points, no differences were observed (Student's *t*-test, $p > 0.1$). Similar results were found in March 2006, where values ranged from 0.08 to 0.26 d^{-1} with no significant differences between the night (0.19 ± 0.09 d^{-1}) and the light period (0.17 ± 0.07 d^{-1}) (Student's *t*-test, $p > 0.9$) (Figure IV-20a). These results confirm the observations in batch cultures of *Skeletonema marinoi* (see section II).

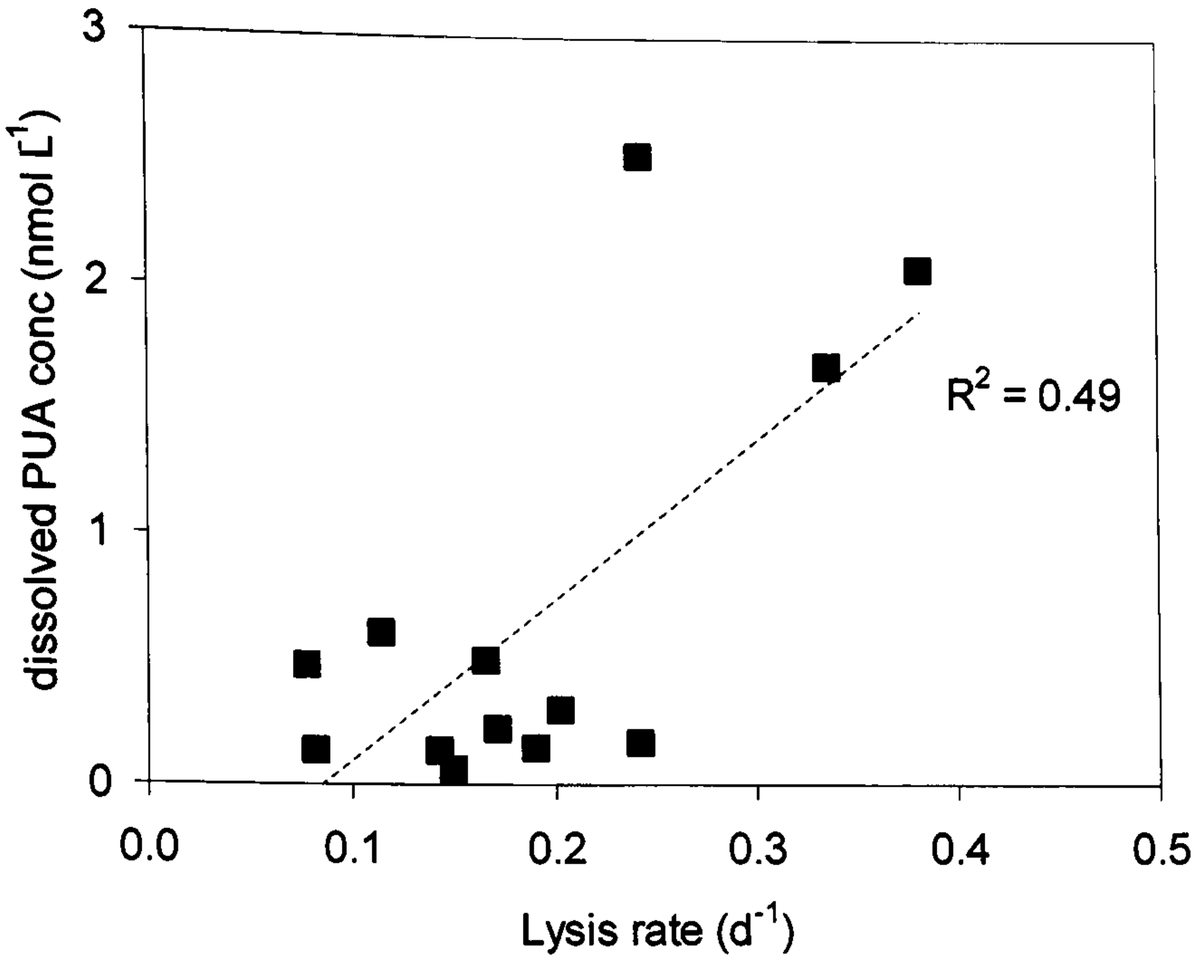


Figure IV-19. Relation between dissolved PUA concentrations (nmol L^{-1}) and lysis rates (d^{-1}) in March 2005 and 2006 (R^2 represents coefficient of correlation of the trend line).

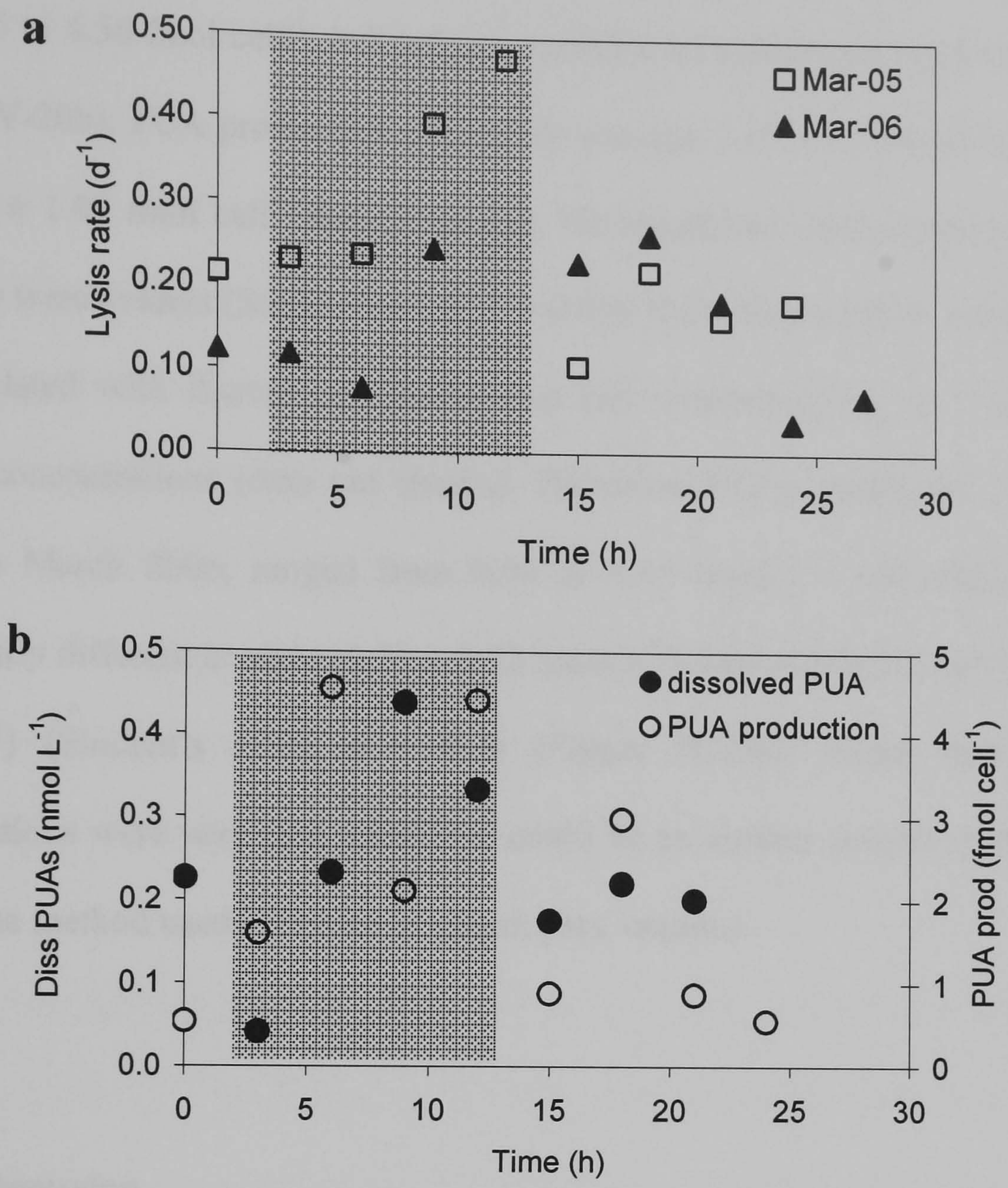


Figure IV-20: a) Lysis rates (d^{-1}) observed over a 24 and 27 h-period during *Skeletonema marinoi* blooms in March 2005 (open squares) and 2006 (black triangles) at a fixed station. Shaded region represents dark period. b) Dissolved PUAs (nmol L^{-1}, black circles) observed over a 21 and 24h-period in March 2006 and potential cell PUA production (fmol $cell^{-1}$, open circles) in March 2005 during *Skeletonema marinoi* blooms at a fixed station. Shaded region represents dark period.

The potential PUA production estimated over a 24 h-period in March 2005 ranged from 0.55 to 4.56 fmol $cell^{-1}$, indicating a variation of almost one order of magnitude (Figure IV-20b). PUA production reached on average 3.17 ± 1.53 fmol $cell^{-1}$ at night and 1.16 ± 1.09 fmol $cell^{-1}$ during the day. No significant oscillations related to the diel cycle were evident (Student's *t*-test, $p > 0.05$). Variations of PUA production were not correlated with diatom or *Skeletonema* cell concentrations, or with inorganic nutrient concentrations (data not shown). Dissolved PUAs, estimated over a 21 h-period in March 2006, ranged from 0.04 to 0.44 nmol L^{-1} and values were not significantly different at night (0.30 ± 0.12 nmol L^{-1}) than during the day (0.17 ± 0.09 nmol L^{-1}) (Student's *t*-test, $p > 0.5$) (Figure IV-20b). Since dissolved PUA concentrations were very low, variations could be an artifact caused by the detection limit of the method used (Thomas Wichard, pers. comm.).

III.4. Discussion

The results presented here indicate that high lysis rates, up to 0.38 d^{-1}, can occur during blooms of *Skeletonema marinoi*. The few data available on phytoplankton growth in the Northern Adriatic Sea indicate that the coastal area is characterized by growth rates of 0.5 d^{-1} (± 1.1 SD d^{-1}) and loss rates of 0.28 d^{-1} (± 1.2 SD d^{-1}), and the off-shore area by growth rates of 0.05 d^{-1} (± 0.30 SD d^{-1}) and loss rates of 0.40 d^{-1} (± 0.40 SD d^{-1}) (Pugnetti et al. 2004). Pugnetti et al. (1994) conclude that the low growth rates, estimated by ^{14}C uptake, are due to high loss processes, such as cell lysis,

respiration and carbon excretion. Together with our results, this may indicate that cell lysis rates during the bloom of *Skeletonema marinoi* is an important factor for the dynamics of diatom populations, and supports the existence of a "non-predatory" death of marine phytoplankton as during the bloom of *Phaeocystis* spp. in the North Sea (Van Boekel et al. 1992; Brussaard et al. 1995; Riegman and Winter 2003). The lysis rates observed during *Skeletonema* blooms are higher than in culture (see section II) and low Chl *a* per cell were found (Chl *a* concentrations divided by *Skeletonema* concentrations) (Table IV-2), which may indicate that the diatom population was at the late declining phase. However, it can not be excluded that the lysis rate estimates were biased by the contribution of other non phytoplanktonic organisms to the total esterase activity (which may lead to overestimates).

Cell lysis rates measured during the winter were high (0.16 d^{-1}) as compared to the North Sea (0.05 d^{-1}) (Van Boekel et al. 1992; Brussaard et al. 1996) while the values measured in June are lower (0.09 d^{-1}) than those observed in the coastal and open Northwestern Mediterranean Sea (0.41 and 0.86 d^{-1}, respectively) (Agusti et al. 1998; Agusti and Duarte 2000), and the North Sea (0.20 d^{-1}) (Riegman and Winter 2003). These authors found highest lysis rates in summer and have therefore associated them to high temperature and irradiance and low nutrient and Chl *a* concentrations. Due to the large nutrient loads from the Po River that are redistributed over a large area of the basin, the Northern Adriatic Sea can be extremely eutrophic in the summer (Boldrin et al. 2005), as observed in June 2004, when high inorganic nutrient and Chl *a*

concentrations were measured; this may explain the lower lysis rates relative to the above mentioned regions.

During the March cruises, Chl *a* concentrations were low (0.26 ± 0.17 μg L^{-1}) while high lysis rates (up to 0.24 d^{-1}) were associated with a high molar C:N ratio (up to 14.4). The same pattern of an increase of C:N with increasing cell lysis was observed in declining cultures of *Skeletonema marinoi* grown in f/2 medium, and has been related to silicon limitation (see Chapter II-B). Low concentrations of silicic acid (< 0.5 μmol L^{-1}) were also found during the *Skeletonema* blooms in March 2002, together with high lysis rates (< 0.22 d^{-1}), which in this case may indicate that silicon limitation was responsible for the decline of the bloom. Casotti et al. (2004) found that a high percentage of apoptotic cells occurred at the peak of the *Skeletonema* bloom in 2002 which may suggest an endogenously-controlled process of initiation of lysis of phytoplankton cells. Apoptosis is a common phenotype of programmed cell death, an active mechanism of cell disruption in response to stress or changes in environmental factors, and there is growing evidence that this process is relevant for phytoplankton (Bidle and Falkowski 2004; Franklin et al. 2006).

Phytoplankton automortality *in vivo* can be exogenously triggered by biotic factors, such as viral (for review Brussaard 2004 and Suttle 2005; Bidle et al. 2007) and bacterial attack (Cole 1982; Mitsutani et al. 1992), and/or abiotic factors, such as nutrient and light limitation (Berges and Falkowski 1998; Segovia et al. 2006). The linkage between the different environmental factors and their potential to cause cell

lysis rates relative to phytoplankton is very difficult to demonstrate in nature since these factors can occur at the same time (Riegman and Winter 2003). Additional complexity is brought by the fact that these factors interact with each other, as found by Brussaard et al. (Brussaard and Riegman 1998) who showed that the magnitude of cell lysis induced by nutrient limitation can be modified by bacteria. Moreover, there is growing evidence that other factors can play an important role in determining algal bloom dynamics and fate, such as the production of algae-derived toxic compounds (Landsberg 2002; Legrand et al. 2003), which are not yet taken into consideration when studying phytoplankton dynamics at sea.

Diatom-derived PUAs have been shown to induce programmed cell death in diatoms (Casotti et al. 2005) and substantial amounts of dissolved PUAs were observed together with high lysis rates in phytoplankton populations dominated by *S. marinoi* (Figure IV-19). These results indicate that PUAs can be released in the water following diatom cell lysis, as observed with freshwater diatoms (Wendel and Juttner 1996) and therefore may be toxic not only for grazers but also for diatoms, acting as a diffusible bloom-termination signal that triggers an active cell death mechanism at sea, as suggested for *Phaeodactylum tricornutum* in culture (Vardi et al. 2006). In addition, substantial amounts of PUAs with high lysis rates were also observed in mixed phytoplankton communities (diatoms and non-diatoms), suggesting a potential toxic effect of these compounds on surrounding species, as found in cultures (see Chap III section 1 and 2). This supports the hypothesis that PUAs may play a role as

allelochemicals by mediating interactions among planktonic organisms and therefore regulating the diversity of these communities.

Preliminary results on dissolved PUAs at sea show concentrations ranging from 0.13 to 2.53 nmol L^{-1} which strongly support the hypothesis that PUAs can be released into seawater following cell lysis. However, it is quite unlikely that these concentrations reflect the amount of PUAs to which nearby organisms are exposed. An average PUA production of 1.27 fmol $cell^{-1}$ was measured during the diatom blooms which would correspond to 1.25, 0.12 and 0.01 µmol L^{-1} at 1, 10 and 100 µm in the surrounding of one single diatom cell, respectively, soon after its lysis (calculated from the random-walk model of molecular diffusion (see Chap III-A equation 3). These theoretical concentrations are in a significant range for affecting the growth rate of diatoms and other organisms (see Chap III). High PUA production during *Skeletonema* blooms were associated with increasing lysis rates (Figure IV-17a) and highest PUA production (6.71 and 10.55 fmol $cell^{-1}$) occurred when concentrations of inorganic nitrogen and phosphate were particularly low (< 1.6 µmol L^{-1} and < 0.01 µmol L^{-1}, respectively). This is in remarkable agreement with *Skeletonema* in culture, where PUA production increases in dying cells and under nutrient stress (see Chap II-B). So, it is reasonable to expect that PUA concentrations at sea are high enough to affect the growth of algae surrounding diatoms.

The highest amount of dissolved PUAs was measured when cell lysis was high (0.24 d^{-1}) but neither *Skeletonema* cells nor PUA production was found, suggesting

that: (1) organisms other than *Skeletonema* can produce and release PUAs in seawater. Phytoplankton cells were harvested on 1.2 μm GF/C filters, thus smaller cells, such as picoplankton and bacteria, probably passed through the filters. Although no PUAs have been detected yet in these organisms (Charles Vidoudez, pers. comm.), we cannot eliminate the possibility that small cells are able to produce PUAs. (2) Another explanation may be that dead *Skeletonema* cells released PUAs a short time before the sampling date and subsequently lysed and disappeared from the counts. This may be less likely because Satchwill et al. (2007) recently found that > 90% of PUAs was lost after 48 h in salty water. (3) Finally, it has been recently found that PUA production was a continuous process that takes place as long as enzymes are in contact with precursor free fatty acids and not retro-inhibited by the amount of PUAs produced (Fontana et al. 2007a). Very little is known about the half-life of the enzymes involved in natural conditions, but preliminary results shows that lipoxygenase, one of the key enzymes for PUA production, is still active 48 h after being released in seawater (Andrea Gerecht, pers. comm.) and therefore free enzymes could be producing PUAs long after cells have disappeared.

III.5. Conclusions

Our results suggest that lysis may be an important loss factor during blooms of *Skeletonema marinoi* in the Northern Adriatic Sea. The effect of nutrient limitation with the toxic effect of dissolved PUAs may trigger cell lysis in particular

circumstances. The presence of dissolved PUAs in seawater supports the hypothesis of the multiple function of PUA production in diatoms as chemical defense against grazers, signal molecules for bloom termination and as allelochemicals against other planktonic organisms, similar to other toxins, such as dimethylsulfide and acrylic acid in prymnesiophytes (Steinke et al. 2002). Obviously, the environmental factors responsible for the trigger and/or the modulation of cell lysis deserves further research efforts.

CHAPTER 5 Conclusions

Diatoms undergo seasonal blooms that attract billions of organisms, from bacteria to predators. The question has arisen as to how these tiny single cells succeed to thrive while being surrounded by myriads of predators, competitors and pathogens. One strategy chosen by diatoms may be represented by the production of defensive chemicals. Some bloom-forming diatom species produce and release reactive PUAs and other hydrocarbon compounds that may act as information-conveying molecules (infochemicals) (Pohnert et al. 2007). It is now accepted that PUAs alter reproduction of grazers, such as copepods, and we provide significant evidence that PUAs may also affect the growth of the competitors (Chapter III section I) and therefore dramatically affect food-web structure and community composition.

Figure V-1 presents a theoretical series of interactions between PUA-producing diatoms and surrounding organisms, such as bacteria, phytoplankton and copepods.

During the early stage of diatom blooms, due to their extraordinary capacity to uptake inorganic nutrients that enables them to divide actively (see Sarthou et al. 2005 and Wilhelm et al. 2006) and to their strong silicified cell wall that provides a mechanical defense against copepods (Hamm et al. 2003), diatoms may outcompete other phytoplankton species and resist herbivory attack, giving rise to dense blooms. During this period of optimal growth conditions, diatoms accumulate photosynthetic storage products, such as PUFAs, that provide substrates for the chemical defense

(chapter II section II) (Figure V-1a). In addition, diatoms may experience high UV exposure, increasing potential PUA production, likely due to an up-regulation of the genes involved in the synthesis of the required enzymes (Bonomelli et al. 2004), thereby increasing the toxicity of diatoms on copepod reproduction (Kouwenberg and Lantoine 2007). During this period of rapid growth, defense against grazers may be mainly provided by mechanical defense, and the few grazers that succeed in breaking the silicified cell wall may show altered reproductive capacity.

After the period of active cell divisions, diatoms slowly experience physiological stress when the major part of the essential nutrients is consumed, thereby triggering the onset of the stationary phase. If silicic acid is the limiting nutrient, this will induce an increase in potential PUA production following cell lysis and the release of PUAs in seawater. The concentrations of PUAs would remain at sub-lethal levels and therefore stimulate the resistance of the diatom population to PUAs, providing an early-warning protective mechanism (Vardi et al. 2006) (Figure V-1b).

Despite the fact that the diatom population experiences silicon limitation, cells are still able to divide, though less actively, by undergoing metabolic adaptations (Whilhelm et al. 2006). On one hand, the effect of such nutrient limitation weakens the diatom cell wall and potentially reduces the cell's capacity to mechanically defend itself against copepods. On the other hand, the cell strongly enhances its chemical defense against grazers (Chapter II section II). In this case, PUA production may represent the main line of defense for the diatom cells, shifting from mechanical to chemical (Figure V-1b).

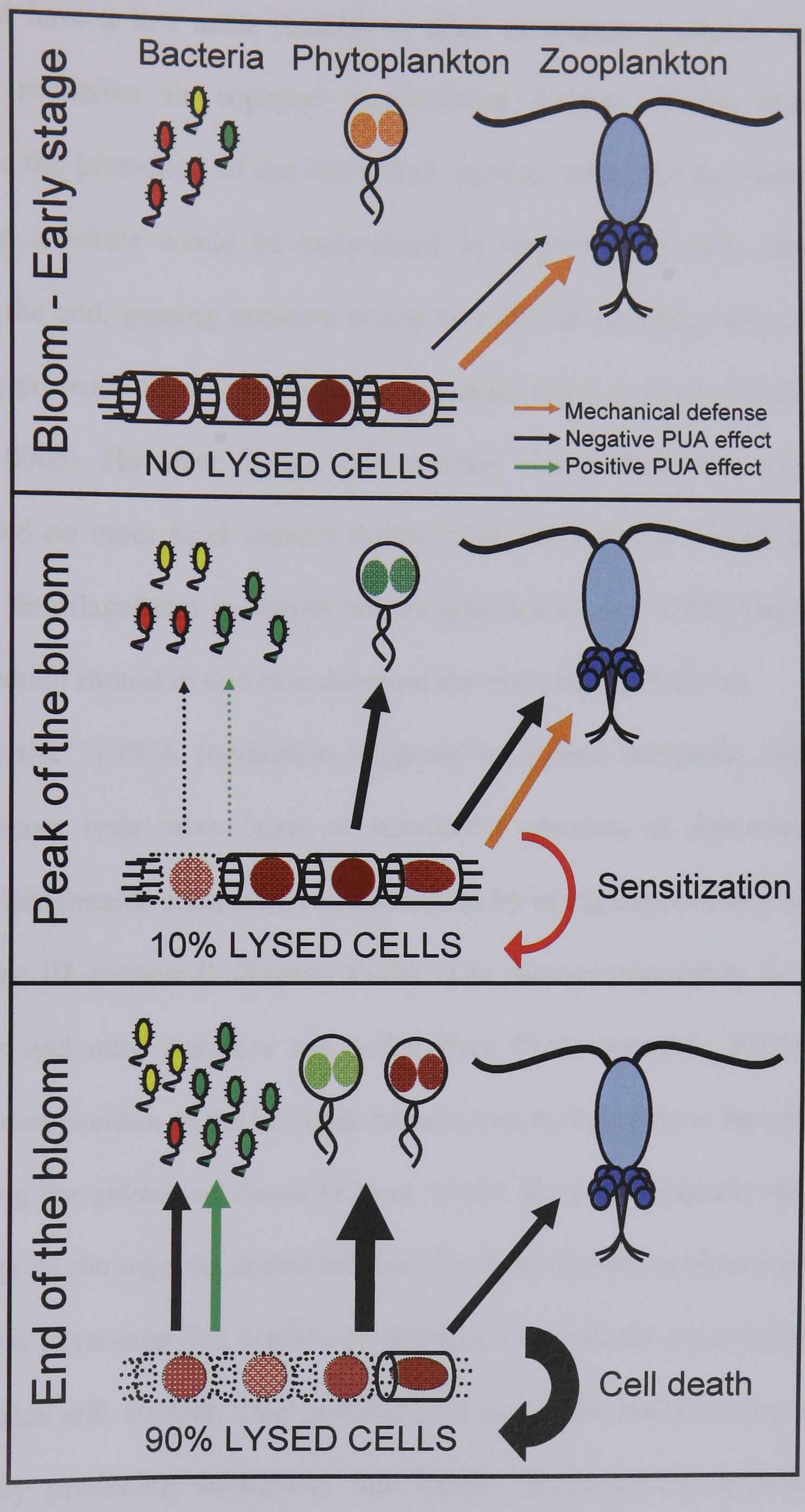


Figure V-1. Possible exposure pathways of heterotrophic bacteria, phytoplankton and zooplankton to diatom-derived polyunsaturated aldehydes.

PUAs that have a low acute toxicity to adult or mature copepods and lead to postdigestive reduction in copepod reproductive success would obviously be ineffective for the protection of the individual ingested cells, but the defence of the community as a whole would be maintained by targeting the next generation of copepods. In the end, grazing pressure would be reduced allowing blooms to persist when grazing pressure would otherwise have caused them to crash (Cembella 2003; Ianora et al. 2006). However, it has been recently shown that some copepods can selectively feed on other food sources during their diel vertical migrations, such as heterotrophic dinoflagellates in deeper waters (Halsband-Lenk 2005; Halsband-Lenk et al. 2005), which should in this case decrease the toxic effect of PUAs

The great rise in PUA production triggered by silicon limitation, together with increasing diatom lysis rates, leads to substantial amounts of dissolved PUAs in seawater. By this means, competitors are inhibited by being exposed to toxic levels of PUAs (chapter III section I) (Figure V-1b). The diatom population is immunized against PUAs and may therefore not suffer from PUA exposure. PUAs may also influence the composition of the bacterial community, by being toxic for some bacteria and stimulating the growth of others (Figure V-1b). Resistant bacteria may therefore take advantage of the organic matter released by dead diatoms and recycle inorganic nutrients. What advantage this confers to diatoms, to select for a particular group of bacteria, remains still unclear. One possibility is that some bacteria may also act as competitors by producing substances that inhibit phytoplankton growth, such as *Cytophaga* sp. on the diatom *Skeletonema costatum* (Mitsutani et al. 1992); these bacteria may in turn be inhibited by PUAs.

Diatom cell lysis rates increase during the decay of the bloom. Hence, the concentrations of PUAs in seawater may sharply increase and reach critical levels for diatoms themselves. At this point, PUA exposure may induce programmed cell death (Casotti et al. 2005), in turn increasing cell lysis rates and PUA release, leading to bloom termination (Figure V-1c). This massive release of PUAs in seawater may have a strong impact on bacterial and phytoplankton communities, selecting only for resistant species. Since other phytoplankton species are available for copepods, it is likely that the alteration of copepod reproduction is minor, as observed in Dabob Bay, Washington, during the decay of blooms of the toxic diatom *Thalassosira pacifica* (Halsband-Lenk 2005).

At a first view, this type of defense might not make evolutionary sense for unicellular algae that do not survive the ingestion process and release allelochemicals following cell death. Nevertheless, this mechanism of defense could be effective at the population level. If PUAs are truly functioning as allelochemicals, then this suggests group selection in addition to the fitness of the population. Group selection acts to increase the fitness of a group of individuals independently of the fitness of each individual in the group (Crespi 2001). A field study that evaluated intraspecies genetic variability in populations of the harmful diatom *Pseudo-nitzschia delicatissima* indicated a high genetic variability in pre-bloom conditions whereas only one major clade dominated during the bloom (Orsini et al. 2002). Therefore, the evolution of a defense mechanism of bloom-forming cells would be possible even if certain

individuals are sacrificed (Wolfe 2000). The demonstration that PUA-producing species can undergo resting stage formation at the end of the bloom, so preserving the PUA-forming genotype, would strengthen this argument. The encysted cells could then in theory reactivate during favorable conditions, and so begin bloom formation once more and benefit from a reduction of the pool of grazers and competitors. This concept lacks experimental verification for now, but molecular analyses of phytoplankton populations may represent a key step to understand the ecological role of these compounds in marine ecosystems.

For evolutionary considerations, the metabolic costs involved in chemical defenses should also be considered. Interestingly, the rapid wound-activated release of polyunsaturated aldehydes depends on processes that are closely associated with primary metabolism (Chapter II section II), such as storage lipids, which do not require the ad-hoc production of cost-intensive secondary metabolites (see Wolfe 2000; Pohnert 2005). It is likely that these lipids, as well as the enzymes involved, are also required for the regulation of cellular processes in intact cells (Pohnert 2005). In this case, the cost of chemical defense might be negligible and the evolution of such a defense mechanism could thus be driven by the need for processes involved in primary metabolism rather than by the need for grazing pressure reduction, which might be an indirect beneficial effect.

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